Host-HIV Interactions *in vivo* and *in vitro*

Dissertation

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Summary

The introduction of highly active antiretroviral therapy has been a major breakthrough in HIV research but is not able to cure infected patients. A detailed understanding of HIV biology is mandatory to develop new treatment strategies.

This thesis explored three aspects of Host-HIV interaction. First, we established a human-to-small animal xenotransplantation model, which is based on the transplantation of $CD34^+$ cord blood cells into newborn $Rag2^{-/-}$ *Il2rg*$^{-/-}$ mice, as a new tool to study HIV infection and pathogenesis *in vivo*. Notably, HIV research has been hampered due to the lack of assessable animal models. Upon CCR5-tropic or CXCR4-tropic HIV challenge, these mice develop long-term, high-titer, and lymphoid organ disseminated infection, closely resembling HIV infection in humans. This straight-forward to generate, cost-effective, ethically unproblematic, and easy to monitor new *in vivo* model should thus be valuable to study virus-induced pathology, as well as pharmacologic or genetic approaches aiming to prevent or treat HIV infection. In particular, these mice may help to investigate vaginal and rectal transmission or the role of chronic immune activation in HIV pathogenesis.

Chronic immune activation is a major cause for progressive immunodeficiency in HIV infection. The underlying trigger, however, remains largely unknown. HIV single stranded RNA (ssRNA) reaches high copy numbers throughout the infection and has been shown to trigger Toll-like receptor (TLR) 7 leading to cellular activation. We used the synthetic compound R848 to explore the effects of sustained TLR7 triggering on the murine lymphoid system. We report immune activation and disruption of the lymphoid system reminiscent of HIV-associated pathology. This suggests that HIV ssRNA may directly contribute to chronic immune activation and dysfunction by signalling through TLR7. Hence, manipulating TLR7 triggering or down-stream signalling may be therapeutically valuable to reduce chronic hyper-immune activation and immune dysfunction.

Third, we investigated whether HIV exploits signalling through the HIV receptor complex (i.e., CD4 and CCR5) to optimize conditions for viral replication. We generated human T-cell lines stably expressing different combinations of either wild-type or mutant CD4 and CCR5 and used them to study both the overall influence of signalling through the receptor complex on HIV replication and the contribution of each receptor to such effects. In conclusion, our data suggest that signalling through CD4 has an inhibitory effect on early reverse transcription, whereas CCR5 signalling does not play a major role, and later steps of the HIV replication cycle are not modulated by CD4/CCR5 signalling.

Zusammenfassung

Die Entwicklung von antiretroviralen Medikamenten war ein bedeutender Meilenstein im Kampf gegen HIV. Dennoch vermögen auch modernste Therapien Patienten nicht zu heilen. Deshalb sind neue Strategien notwendig. Dies verlangt ein vertieftes Verständnis der Biologie von HIV.

Den Forschern fehlte bis anhin ein geeignetes Kleintiermodell. Im ersten Teil dieser Arbeit wurde ein vielversprechendes neues HIV Mausmodell etabliert. $CD34^{+}$ blutbildende Vorläufer- und Stammzellen aus Nabelschnurblut wurden $Rag2^{-/-}$ $Il2rg^{-/-}$ Mäusen unmittelbar nach Geburt intrahepatisch verabreicht. Daraufhin liessen sich diese mit CCR5 und CXCR4-tropen HIV Stämmen infizieren, entwickelten eine hohe Virämie in allen lymphatischen Organen und zeigten einen ähnlichen Infektionsverlauf wie im Menschen. Die pathobiologischen Prozesse von HIV sowie neue therapeutische oder genetische Behandlungsansätze können nun mit unserem Modell untersucht werden. Besonders für das Studium der vaginalen und rektalen Transmission oder der Bedeutung der chronischen Immunaktivierung sollte dieses Modell hinreichend geeignet sein.

Immunaktivierung wird als eine der Hauptursachen der progressiven Immundefizienz im HIV Patienten verstanden. Der Auslöser ist aber nach wie vor unklar. Kürzlich wurde *in vitro* gezeigt, dass einzelsträngige HIV RNA (ssRNA) über die Stimulation von Toll-like Rezeptor (TLR) 7 direkt Immunaktivierung induziert. Wir zeigten nun in der Maus, dass die repetitive Stimulation von TLR7 mittels der synthetischen Droge R848 zu vergleichbarer Immunaktivierung und Absonderlichkeit des lymphatischen Systems führte, wie sie im HIV infizierten Patienten beobachtet wird. Wir schliessen daraus, dass die ständige Stimulation von TLR7 durch HIV ssRNA, welche während der ganzen Infektionsdauer in hoher Zahl vorhanden ist, eine direkte Ursache von Immunaktivierung und Immuno-adenopathie der HIV Infektion sein könnte. Ein therapeutischer Eingriff in die TLR7 Signaltransduktion zur Eindämmung der Immunaktivierung sollte getestet werden.

HIV gelangt nach Bindung an den CD4/CCR5 Rezeptor-Komplex in die Wirtszelle. Wir generierten rekombinante T-Zelllinen, die verschiedene Kombinationen von natürlichem und mutiertem CD4/CCR5 exprimieren. Damit untersuchten wir im dritten Teil ob Signal-transduktion über den Rezeptor Komplex die HIV Replikation positiv oder negativ beeinflusst. Unsere Daten legen nahe, dass die frühe reverse Transkription durch CD4 Aktivation unterdrückt wird, wohingegen CCR5 Aktivation nur geringfügige Auswirkungen hat. Ebenso scheinen spätere Schritte des Replikationszyklus unbeeinflusst.

1

Introduction

Chapter 1.1, 1.2, and 1.3 provide an introduction into HIV biology. Chapter 1.4 and 1.5 focus on recent progress in the area of HIV animal models, particularly mouse models transplanted with human lymphoid tissue. Chapter 1.6 describes recent advancement in the understanding of immune activation and HIV pathogenesis and chapter 1.7 discusses the impact of HIV receptor complex signalling on HIV replication.

1.1 HIV Infection – Global impact

Since the beginning of the human immunodeficiency virus type-1 (HIV) pandemic, 25 years ago, HIV infected 65 million people and killed 25 million! Each year, more than 4 million people are newly infected worldwide. To date, the number of HIV-infected people is estimated at 40 million [1]. In 2001, leaders from 189 UN Member States recognized that HIV infection was one of the most urgent issues of national and international development. They signed the "Declaration of Commitment on HIV/AIDS" to help reach the "Millennium Development Goal" of stopping and beginning to reverse the HIV epidemic by 2015 [1].

Since its introduction, antiretroviral therapy (ART) has resulted in marked decreases of morbidity and mortality of patients infected with HIV. However, ART has substantial limitations such as side effects and emergence of drug resistant virus strains. Most notably, the high cost of ART restricts its usage nearly exclusively to the 1st world. Thus, the HIV pandemic continues, presenting new medical problems and leading to almost 3 million deaths in 2005 [1]. Novel treatment strategies are urgently needed to reach the ambitious aims of the above mentioned declaration. Defining potential targets for intervention requires a detailed understanding of the HIV biology. This in turn necessitates appropriate *in vitro* and *in vivo* models.

1.2 Genetic organization, structure, and replication cycle of HIV

HIV is a member of the genus *Lentiviridae* and belongs to the family of the *Retroviridae* which are characterised by their unique replication cycle: genomic RNA containing viral particles are reverse transcribed upon entry into the host cell and, thereafter, integrated into the host genome. The integrated viral genome, the provirus, is replicated along with the host cell DNA and serves as a template for transcription of viral RNA, which subsequently is translated into proteins eventually resulting in the formation of progeny virions.

The HIV genome is 9.7 kb in length and encodes the *gag* (group-specific antigen) *pol* (polymerase) and *env* (envelope) genes yielding the nucleocapsid core proteins, enzymes required for replication, and envelope proteins (Fig. 1). *Gag*, *pol*, and *env* are conserved in all retroviruses. HIV, in addition, encodes three early, mostly regulatory, and three late accessory proteins. The early proteins Tat (transactivator of transcription), Rev (regulator of virion gene

expression) and Nef (negative effector) play a major role in controlling gene expression. Notably, Tat is a powerful transactivator of gene expression, which acts by both inducing chromatin remodeling and by recruiting transcriptional complexes. Rev protein acts postranscriptionally and regulates the transition between the early and late phases of viral gene expression by allowing the transport of singly spliced and unspliced viral mRNA species from the nucleus to the cytoplasm. Although Nef was originally named "negative factor," it has a positive role in viral replication and pathogenesis. Multifunctional Nef helps the virus to maintain high viral loads and to overcome host immune defenses by interacting with host cell signal transduction proteins to provide for long term survival of infected T cells and by advancing endocytosis and degradation of CD4 and MHC proteins, which possibly impairs cytotoxic T-cell function.

The accessory proteins Vif (viral infectivity factor), Vpr and Vpu (viral protein r, u) have multiple functions, i.e., RNA processing, virion assembly, and host gene expression [2]. The long terminal repeats (LTR) on both sides of the genome are regulatory elements and do not code for any proteins.

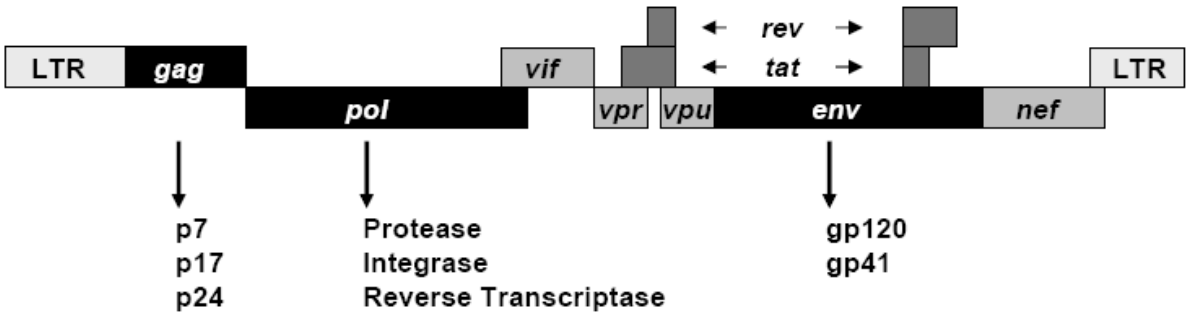

Fig. 1. Genetic organization of HIV. The three major genes *gag*, *pol*, and *env* (shown in black) encode polyprotein precursor proteins that are subsequently processed by viral or cellular proteases into mature proteins (indicated below). Gag is processed into capsid and matrix proteins. Pol is processed into protease, reverse transcriptase and integrase. Env is cleaved by cellular proteases into gp120 and gp41. In addition HIV encodes regulatory and accessory genes and LTRs, which contain various regulatory protein binding sites. Abbreviations are explained in the text.

A mature HIV virion is made of envelope, capsid, matrix, and enzyme proteins (Fig. 2). The viral envelope derives from the host cell and thus contains some host-cell membrane proteins and in particular the HIV envelope glycoprotein Env. Env consists of an outer protruding cap glycoprotein (gp) 120, and a transmembrane protein gp41, which are non-covalently linked to each other forming a heterodimer which is present on the virion surface as a trimer. The inner surface of the virus is made of the matrix protein p17 and a viral core or capsid, comprising the capsid protein p24. The major elements contained within the HIV capsid are two single strands of unspliced HIV genomic RNA, which are stabilised as a ribonucleoprotein complex with the nucleocapsid protein p7, and three enzyme proteins, reverse transcriptase, protease and integrase.

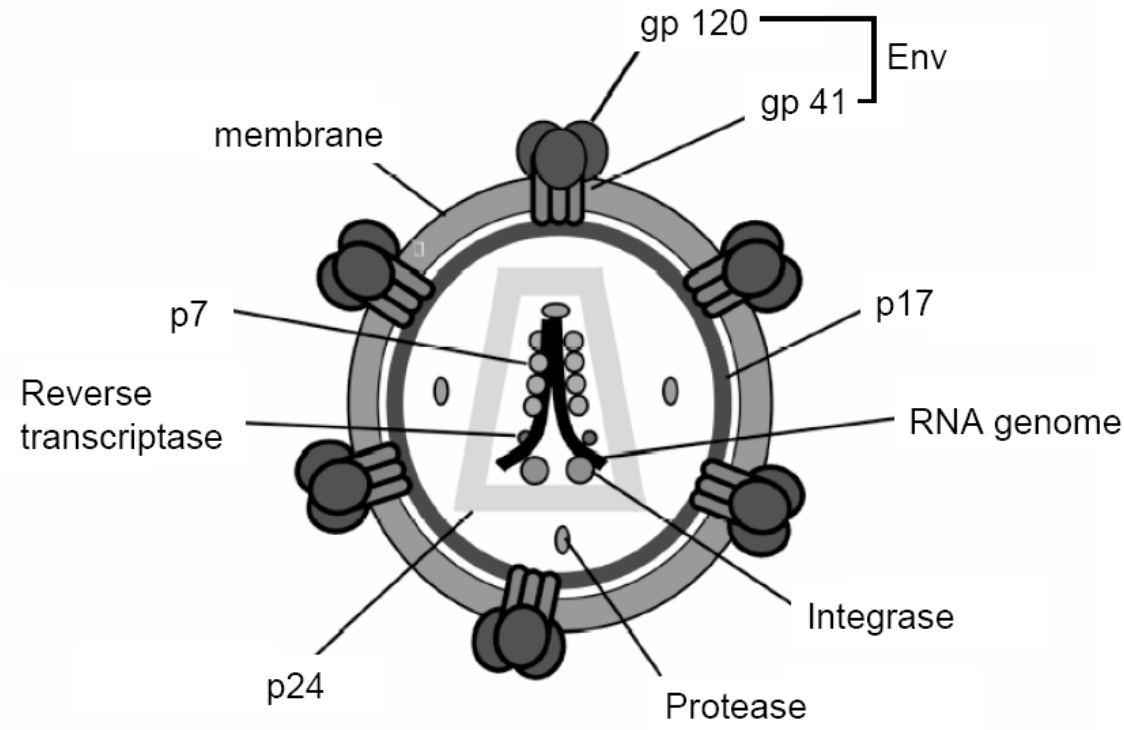

Fig. 2. Structure of HIV. Abbreviations are explained in the text.

The binding of the HIV Env protein mediates virus entry and initiates the viral replication cycle (Fig. 3). Gp120 binds to the cell-surface receptor CD4 causing a conformational change in the gp120 molecule resulting in the engagement of a second receptor, i.e., CCR5 or CXCR4. CCR5 and CXCR4 are also known as HIV co-receptors. They are seven-

transmembrane receptors and belong to the family of chemokine receptors. A subsequent change in gp41 eventually leads to the fusion of the HIV envelope and the cell membrane, enabling the viral core to enter the cell. In the cytoplasm, HIV reverse transcriptase converts viral RNA into complementary DNA (cDNA) which is transported as preintegration complex to the nucleus. In the nucleus, the integrase covalently integrates the viral DNA into the host genome creating the provirus. The provirus either remains latent or produces progeny virus by synthesis of viral mRNAs, which are transported to the cytoplasm for translation. Newly made core proteins, enzymes, and genomic RNA assemble in the cytoplasm and form an immature viral particle. This particle buds of from the cell and acquires its envelope from the cell membrane. Released particles complete maturation and become infectious.

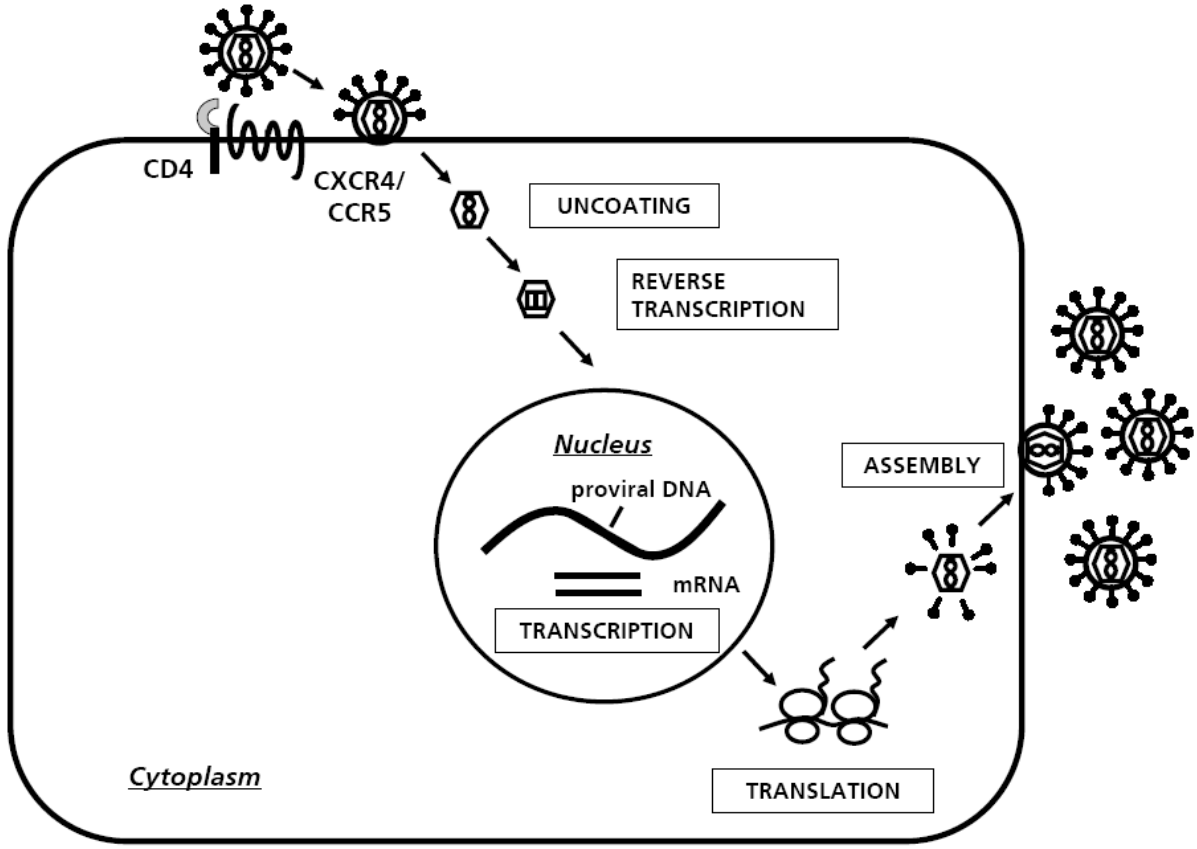

Fig. 3. The HIV replication cycle in $CD4^+$ T-lymphocytes. Steps in viral replication cycle are 1) binding to the cellular receptor complex, 2) fusion and uncoating, 3) reverse transcription, 4) nuclear import and integration into host genome, 5) transcription and translation, 6) assembly, budding and maturation.

1.3 HIV Infection and AIDS

Hallmark of HIV infection is the progressive depletion of $CD4^+$ T cells, also called T helper (T_H) cells. The $CD4^+$ T cells have a crucial role for an efficient immune defense. Thus, in advanced stage of HIV infection patients are at risk to suffer from opportunistic infections, neoplastic diseases, HIV-associated wasting and eventually death [3] . The advanced stage is best known under the acronym "acquired immunodeficiency syndrome" (AIDS). For instructive purposes, the prototype HIV infection can be divided into acute infection, chronic infection and the AIDS stage (Fig. 4): 1) the acute or primary infection is characterized by an initial burst of HIV viremia and sharp decline of $CD4^+$ T-cells. 40-90% of individuals in the acute phase develop a flu-like disease. Within 3 months after transmission, the immune response is able to contrain partially HIV which is mirrored by a recovery of $CD4^+$ T cells and a levelling of the HIV RNA in the range of $5x10^3$ – $1x10^5$ copy numbers per ml; 2) The chronic or asymptomatic phase follows which is also called clinical lateny. The virus replicates, $CD4^+$ T-cell counts continue to drop while the patient is asymptomatic. This phase lasts approximately 10 years. There are, however, patients suffering from rapid progression with only 3 years of clinical latency while on the opposite, there are elite controllers with long-lasting host-endogenous control of HIV; 3) An individual is considered to have AIDS (according to the 1994 expanded World Health Organization AIDS case definition) if a test for HIV antibody gives a positive result, and 1 or more of the following conditions are present:

- > 10% body weight loss or cachexia, with diarrhoea or fever, or both, intermittent or constant for at least 1 month, not known to be due to a condition unrelated to HIV infection.
- cryptococcal meningitis
- pulmonary or extra-pulmonary tuberculosis
- Kaposi's sarcoma
- neurological impairment that is sufficient to prevent independent daily activities, not known to be due to a condition unrelated to HIV infection (for example, trauma, or cerebrovascular accident).
- candiasis of the oesophagus (which may be presumptively diagnosed based on the presence of oral candiasis accompanied by dysphagia.
- clinically diagnosed life-threatening or recurrent episodes of pneumonia, with or without etiological confirmation
- invasive cervical cancer

The acquired immunodeficiency syndrome (AIDS) usually occurs if $CD4^+$ T-cell counts drop below 200 cells/mm^3 blood. Below this level is a substantial risk of opportunistic infections. HIV-infected patients eventually die of opportunistic infections or of HIV-associated cachexia.

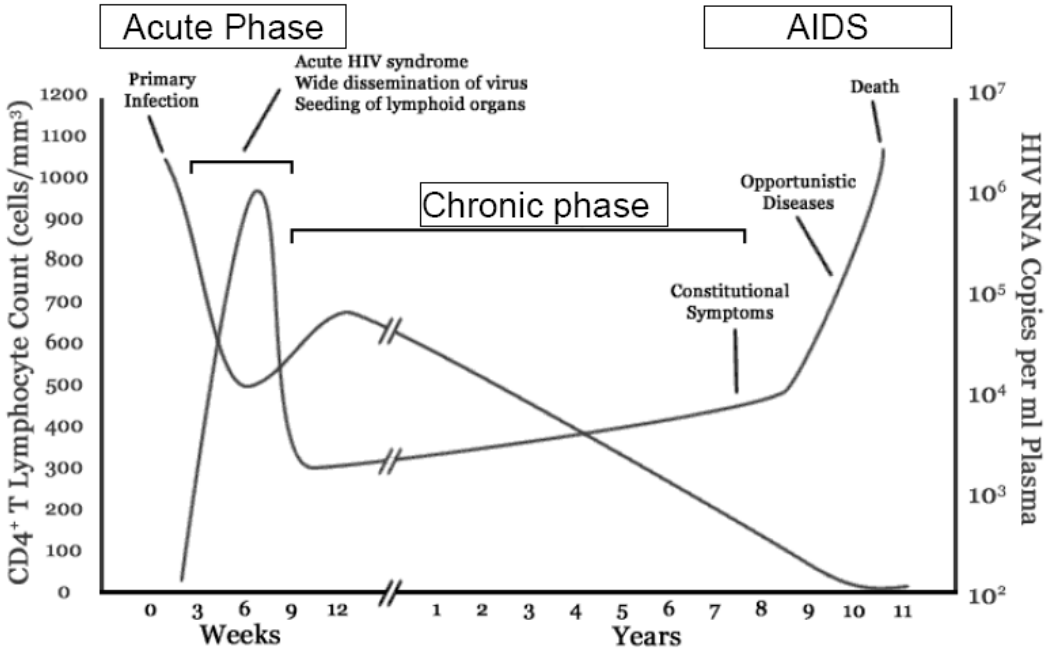

Fig. 4. Prototype HIV infection. During the acute phase, HIV disseminates widely in the body often associated with a marked loss of CD4+ T-cells. Immune responses to HIV lead to decreased viremia. An asymptomatic phase of persistent viral replication, that can last from months to years, follows. When CD4+ T cell numbers decline below a critical level, cell-mediated immunity is lost leading to death. Adapted from [4].

HIV isolates are classified based on their co-receptor use. R5 strains of HIV use CC-chemokine receptor (CCR5) as their co-receptor and can therefore, enter macrophages, dendritic cells and T cells. X4 strains use CXCR4 as a co-receptor and can infect $CD4^+$ T cells only [2]. R5 isolates are responsible for viral transmission, predominate during the early stages of the disease, and are present throughout the whole infection period [5]. Individuals homozygous for a 32–base pair deletion in the coding region of CCR5 are almost completely resistant to HIV infection [6]. In late stage disease, X4 viruses emerge in approximately 50% of all patients. This emergence is associated with an acceleration of the $CD4^+$ T-cell loss. However, it remains controversial whether the emergence of X4 strains is the cause or the consequence of the accelerated immunodeficiency.

1.4 Animal models in HIV research

In vitro cell biology and virology have significantly advanced the understanding of HIV disease. Nevertheless, *in vivo* models are required to address complex questions such as immunopathogenesis and to test potential novel therapies as well as to examine drugs aiming to block HIV transmission. HIV research, however, has been hampered because of the lack of a small animal model that mirrors infection in humans. HIV is a human-specific virus and does not infect or cause disease in other species. Thus, mice or rats, the commonly used animals in biomedical research, are not susceptible to HIV [7-9]. Optimally, an animal model for studying HIV disease would mirror the multifaceted pathogenesis in man, have a well-characterized immune system, and be readily available at low cost. To this end, several animal models for HIV have been established and include monkeys infected either with HIV, simian immunodeficiency virus (SIV) or chimeric HIV-SIV, cats infected with feline immunodeficiency virus (FIV), immunocompromised mice transplanted with human lymphoid tissue as well as transgenic mice [10,11]. In this chapter, I will discuss the animal models that are more frequently used and thus have added substantially to the progress in HIV research - i.e., non-human primates and rodents (especially immunocompromised mice transplanted with human tissue).

1.4.1 Non-human primate models

Chimpanzees and gibbon apes are susceptible to HIV. However, they do not develop HIV-associated immunodeficiency and do not get sick [12,13]. Importantly, these apes are extremely costly for experimentation. For those reasons, chimpanzees and gibbon are rather rarely used.

African green monkeys, sooty mangabeys, rhesus macaques and baboons are exclusively susceptible to SIV [14]. Based on their genetic, antigenic, and biologic properties, SIV is closely related to HIV [15]. Naturally SIV infected African green monkeys and sooty mangabeys remain asymptomatic throughout their life and do not develop any disease despite high level viremia [16].

In contrast to naturally SIV infected monkeys, experimental infection of non-natural host species such as macaques results in a disease pattern that is remarkably similar to human AIDS [17-19]: macaques show diarrhoea, weight loss, hematologic abnormalities including

lymphopenia and thrombocytopenia, lymphadenopathy / lymphoid hyperplasia that progresses to lymphoid depletion, immunosuppression with marked reduction in $CD4^+$ cells and in the $CD4^+/CD8^+$ cell ratio, and opportunistic infections. The majority of macaques infected with SIV die from an AIDS-like disease within one to three years of infection [15]. Thus, the similarity of HIV and SIV pathogenesis, makes SIV infected macaques very attractive for biomedical research [10]. In addition, the investigation of the mechanisms that account for the non-pathogenic course of infection in naturally SIV-infected monkeys provided important clues to the pathogenesis of immunodeficiency in HIV-infected humans [20].

However, there are clear limitations for using the SIV monkey model: they are extremely costly with ~5,000 - 12,000 $ per macaque, limited availability, and ethical concerns. There is a particular concern and uncertainty about the acceptability of using non-human primates in medical research, primarily because of their evolutionary proximity to human beings. The report "Forschung an Primaten – eine ethische Bewertung" from the Federal Animal Experimentation committee evaluated the ethical position concerning monkey experiments in Switzerland and recommended to authorize experiments with utmost reserve [21]. Further and very crucial, and even though SIV is closely related to HIV, the results obtained with SIV in monkey models cannot be directly extrapolated to HIV: for example, the accessory protein Vpu does not have a homologous counterpart in SIV. Another example is the virus-specific difference in reverse transcriptase (RT): the non-nucleosid RT inhibitors which are used in daily clinical practice inhibit HIV RT but not SIV RT [22,23]. SIV/HIV chimeric viruses (SHIV) have been developed to overcome some of these limitations and have been useful for *in vivo* testing of numerous vaccines that specifically target HIV proteins. Unfortunately, SHIV with genomic information mainly from HIV, which would reflect the biological properties of HIV more accurately, were poorly replicating in rhesus macaque cells [23,24].

To summarize, HIV research in non-human primates remains restricted by biological as well as by ethical and financial constraints.

1.4.2 Rodent models

There are significant advantages in developing rodent models over primate models: i) wide availability and low costs, ii) ability to generate large cohort sizes, iii) relative ease of transgenic manipulation [25]. HIV, however, does not efficiently replicate in wild-type mice or rats. In one publication, Langley et al. reported that HIV replicated in non-transgenic cotton

rats [26]. HIV was passaged from animal to animal for three serial passages, but HIV replicated poorly *in vivo*, was only detectable as proviral DNA, and never exceeded one provirus per 1.8 x 10^5 PBMCs. However, it appears that this rat model has not been used anymore since this initial report.

The failure of viral replication in rodents is explained by the lack of appropriate receptors for HIV cell entry, inefficient provirus gene expression and viral assembly in murine cells [25], the block of HIV virus entry and impaired viral gene expression in rat cells [27], and the block of HIV virus entry in rabbit cells [28].

1.4.2.1 Transgenic animals and viruses

To overcome viral integration and replication blocks, investigators have made a huge effort to generate rodents transgenic for known critical host cellular factors. While *in vitro* rodent cells complemented with human trangenes were susceptible to HIV, transgenic rodents were still rather resistant to HIV. For example, Speck et al. overcame the entry block by transfecting rabbit cells with human CD4 and CCR5 *in vitro* and demonstrated that these cells allow viral entry and replication [29]. Transgenesis of rabbits, however, is cumbersome. Thus, this model is buried in oblivion. Keppler et al. overcame the entry block in transgenic rats expressing human CD4 with either human CCR5 or CXCR4. These rats displayed a transient and very low-level plasma viremia upon HIV infection in the range of hundred copies of HIV RNA/ml [30]. Furthermore, HIV replication in these rats occurred only in macrophages and was completely absent in $CD4^+$ T cells for unknown reasons [30].

The situation is similar in mice: the block to HIV entry was overcome in CD4/CCR5 and CD4/CXCR4 transgenic animals. Follow-up studies, however, concurrently indicated that additional post-entry blocks to viral replication must exist. Reduced viral production in murine cells has been attributed to decreased activity of HIV regulatory genes such as *tat* [7] and *rev* [31]. Cyclin T is a species-specific cellular factor recently identified in human cells potentiating Tat activity when transfected into murine cells [32]. Finally, a recent report uncovered an additional cellular block to virion assembly [33]. All these blocks to viral replication must be conquered before the transgenic mouse models can be fully utilized. It is highly unlikely that transgenic mice expressing multiple human genes will represent the long-awaited model.

Alternatively, investigators have adapted the host to express the full HIV genome or some viral genes. Hanna et al. created transgenic mice encoding the entire HIV genome in $CD4^+$ T-cells. These mice developed a severe AIDS-like disease leading to early death [34]. Numerous subsequent studies created mice containing various HIV derived genes. This approach allowed close examination of individual HIV genes and mechanisms that contribute to HIV disease. Joliceur's group, for example, showed that Nef is necessary and sufficient to induce pathogenesis [35]. However, these models can not model the processes of infection such as viral spread and the emergence of viral variants.

Similarly to the approach generating SHIV, murine leukaemia virus (MuLV)/HIV chimeric viruses have been developed to overcome some of the above mentioned limitations. Potash et al. replaced the coding region of the envelope protein gp120 in HIV with that of gp80 from ecotropic MuLV, a retrovirus that infects only rodents [36]. Adult, immunocompetent mice were readily susceptible to infection and virus was detected in splenic lymphocytes, peritoneal macrophages, and the brain. This model has been valuable to study HIV therapy [37], vaccines [38], and neuroinvasiveness [36]. Unfortunately, these chimeric viruses have the same disadvantages as SHIVs – i.e., results obtained with (MuLV)/HIV cannot be directly extrapolated to HIV because of virus-specific differences.

1.4.2.2 "Humanized" mouse models

As outlined above, efforts to genetically engineer rodents to render them susceptible to HIV have largely failed. Even if infection *in vitro* was achieved by human transgene complementation, HIV replication *in vivo* was limited or absent. Thus, substitute xenochimeric mouse models have been developed by transplanting immunodeficient mice with pieces of human cells or tissues that normally serve as targets for HIV. More-recent advances have allowed reconstitution of apparently functional human lymphoid organs, which dramatically enhanced the utility of humanized mice in HIV research.

1.4.2.3 The development of humanized mice

The mice used for engraftment of human tissue must be strongly immunosuppressed otherwise the transplanted tissue will be rejected. The detailed history and state of the art in

this field has been extensively reviewed [39-42]. Briefly, three major breakthroughs have occurred in the field of engrafting foreign tissue in mice which is based on the discovery of distinct mutations resulting in strong immunosuppression:

First, the discovery of the *Prkdc*^{scid} (protein kinase, DNA activated, catalytic polypeptide; severe combined immunodeficiency; abbreviated as SCID) mutation which leads to a complete absence of mature T and B cells allowed researchers to successfully transplant human cells and tissues into mice [43]. However, these mice still had active natural killer (NK) cells, intact innate immunity and spontaneous rearrangement of T- and B-cell receptors over time leading to the generation of mature T and B cells. These limitations explain the rather low engraftment levels of transplanted lymphoid tissue. Preparative treatment or conditioning is mandatory prior to transplantation. Total body irradiation with sublethal doses of γ-radiation is commonly employed. SCID mice, however, have an increased radiosensitivity; the defect in the mechanism for repairing DNA double-strand breaks also leads to a decreased recovery from general radiation damage [44]. The *Rag1* and *Rag2* (recombination-activating gene 1 or 2) mutation prevent mature T- and B-cell development too, but do not cause leakiness or radiosensitivity [45]. Nevertheless, these mice also showed very low engraftment levels of foreign lymphoid tissue; the NK cell activity was claimed to prevent a better engraftment.

The second important event in the development of humanized mice was the generation of non-obese diabetic (NOD)-SCID mice. These mice have lower NK-cell activity and additional defects in innate immunity such as functionally less mature macrophage populations and reduced serum haemolytic complement activity, and supported 5-10 fold higher engraftment levels [46]. Thus, NOD-SCID mice became the gold standard. The relatively short life span of only 8.5 months, because of a high incidence of thymic lymphomas, limited long-term experiments. Unfortunately, the majority of human T- and B-cell do not maturate in these mice.

The third important step was the development of the *Il2rg* (interleukin-2 receptor γ-chain locus; also known as the common cytokine receptor γ-chain, γc, common to the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors) mutation which allowed much higher engraftment rates than in all previously models when crossed to NOD-SCID [47,48] or Rag2$^{-/-}$ mice [49]. Cytokines that signal through γc play a pivotal role in lymphopoiesis [50]. Notably, all *Il2rg* knockout strains completely lack mouse T, B, and NK cells, have normal life spans and show no obviously high rate of tumour development. Many *Il2rg*$^{-/-}$ strains were developed mainly

differing in the *Il2rg* mutation (truncated versus complete absence of IL-2R γ-chain) and strain background. Importantly, we and others found that strain background can severely affect engraftment: BALB/c but not C57BL/6 *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ can be successfully engrafted.

As compared with previously available models, reconstitution of newborn mice represented a major improvement in the field of haematopoietic stem cell (HSC) transplantation [40,49,51]. At the newborn age the expansion of the hemato-lymphoid system is maximal. Transplanting human HSC in newborn mice resulted in the generation of a thymus of human origin with intrathymic *de novo* human T-cell development [49]. The route of injection may also affect engraftment efficiency by directing HSCs to a supportive niche. Intravenous injection increased HSC engraftment as compared to conventional intraperitoneal (i.p.) injection [39]. Intrahepatic injection, as the liver contributes to perinatal hematopoiesis, was even superior [49]. Intra-bone-marrow and *in utero* injections aiming to place HSCs directly into a supportive microenvironment have also been performed [52,53]. The optimal host strain, age and route of infection, however, are not yet known. A thorough direct comparative analysis of the various model systems would need to be done.

Irrespective of the distinct approaches outlined above, the injection of HSCs into sublethally irradiated newborn NOD-SCID *Il2rg*$^{-/-}$ or BALB/c *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice resulted in formation and structuring of major hemato-lymphoid organs and successful differentiation of human cells into many lineages of haematopoietic cells including dendritic cells, natural-interferon producing cells, immunoglobulin producing B cells, T cells, and, to a lower extent, NK cells, myelo-monocytic cells, platelets, red blood cells, and hepatocytes [42,49,51,54,55]. We would like to emphasize that T cells did not reproducibly develop in previous models.

1.5 “Humanized” mouse models with HIV infection

The above mentioned humanized mouse models gave rise to several HIV mouse models. Basically, although many variations exist, two major models have prevailed: 1) the classical SCID-hu and the hu-PBL-SCID models and 2) several more-recent models that rely on the transplantation of HSCs.

1.5.1 The SCID-hu model

The SCID-hu model for studying HIV was developed in 1988 by Mike McCune's group [56-58]: human fetal thymus and liver obtained from fetuses of ~14-23 gestational weeks are surgically implanted under the kidney capsule forming a conjoint organ referred to as thy/liv implant. The thy/liv implant resembles a normal human thymus displaying thymic architecture and an appropriate array of thymocytes subsets allowing thymopoiesis from haematopoietic precursor cells for up to one year [58]. However, there is no or only limited egress of mature T cells from the thymus and no repopulation of human cells from the implant into other organs [59]. Successful HIV infection is only achieved by injecting HIV directly into the implant. Thus, the resultant HIV infection and pathology is restricted to the implant. The levels of human T cells in peripheral blood (~0.7%) and in the spleen are insufficient for disseminated HIV infection. Furthermore, since thymocytes express CXCR4, but only a low level of CCR5, thymocytes are mainly permissive to CXCR4-tropic HIV strains [60]. Notably, in man CCR5-tropic strains are the strains that are predominantly sexually transmitted and persist during early to late stage disease [5,61]. Moreover, monitoring of HIV infection requires either repeated biopsies from the implant or that the animal has to be sacrificed. In particular, it must be noted that neither HIV viremia nor immune responses occur [9,62].

Several modifications to increase the population of human cells were introduced including increasing the quantity of tissue implanted or transplanting additionally human fetal bone marrow [63] resulting in two major advantages: disseminated HIV infection and successful infection when inoculating HIV i.p..

These dynamic systems have been very valuable to study HIV induced pathogenesis such as mechanisms of HIV-induced $CD4^+$ T-cell depletion or the role of HIV accessory genes *in vivo* [58]. Notably, *nef* is critical for achieving high viral loads and depletion of $CD4^+$ T cells [64]. In contrast, *vpu, vpr* or *vif* demonstrated little impact on viral replication or cyopathicity [65]. The SCID-hu models is also useful for the assessment of drug [64] and potentially gene therapeutic strategies [66].

However, none of these modified models was able to mount a primary immune response. Recently a SCID-hu model has been described where fetal thymus, liver, bone marrow, lymph node and skin have been transplanted [67]. In this modified SCID-hu model, primary immune responses have been observed. For practical reasons i.e., obtaining and transplanting all this

fetal tissue, this model will most likely not be generally available for medical research. In fact after its first description in 2000, no further reports have been published.

To summarize, SCID-hu thy/liv mice permit the succesfull HIV infection of the implant. However, HIV pathology is mainly limited to the tissue implant, with naïve T-cells being preferentially susceptible to CXCR4-tropic strain infection. Furthermore, the availability of human fetal organs is very restricted in many countries depending on the legal regulations of abortions (e.g., abortions are not permitted after the 14th week of gestation in Switzerland).

1.5.2 The hu-PBL-SCID model

Human peripheral blood mononuclear cells (PBMC) are widely available; there are no ethical hurdles for getting it. Donald Mosier's group reported the successfull transplantation of PBMC into SCID 18 years ago mice [68]: after i.p. injection there is survival and expansion of human T cells, B cells, NK cells and monocytes. However, human cell numbers in the peripheral blood and other lymphoid organs are low. No human cells are found in the thymus.

Successfull HIV infection was achieved with either cell-free or cell-associated HIV by i.p. injection. Some animal remained persistently infected for 16 weeks. HIV was detected in plasma, spleen, peritoneal lavage, PBMC, bone marrow and lymph nodes [10].

PBMC were also transplanted into NOD-SCID mice as soon as they became available. In these mice higher levels of HIV replication were achieved due to more efficient grafting and proliferation of human T cells [69,70]. Nonetheless, NOD-SCID mice lack potent human antigen presenting cells (APC) such as dendritic cells. The separate injection of human dendritic cells recently improved the model's value for vaccine testing: partial protection from HIV infection, via both humoral and cellular immune responses, was conferred by injecting autologous dendritic cells transduced with replicative defective herpes simples virus amplicons that express HIV gp120 [71].

Massive and systemic HIV infection was reported as soon as NOD-SCID *Il2rg*$^{-/-}$ entered the field. For the first time, HIV viremia reaching as high as 10^6 copies/ml were achieved [72]. This unprecedented susceptibility of the implanted human PBMCs stemmed from unnatural hyperactivation of the PBMCs.

However, there are major disadvantages when transplanting adult PBMC into mice: T cells show signs of major activation within hours and the memory T cells are selectively expanded. In addition, there is no continuous cell renewal because there is no repopulation of the thymus, the spleen or the bone marrow with $CD34^+$ HSC. Very importantly, ~1 month after transplantation, PBMC expand as a consequence of a xenoreactive response and human T cells become anergic and are unable to respond to mitogens or to CD3 stimulation [73]. Most of the transplanted mice show signs of graft-versus-host reaction. Thus, the time span to do experiments is very short.

In summary, immunosuppressed mice transplanted with PBMC sustain HIV infection and replication *in vivo*. However, xenoreactivity and successive loss of human leukocytes limit infection to a relatively short time frame. Moreover infection is skewed toward HIV strains with the CCR5 co-receptor tropism - possibly driven by activation-induced CCR5 expression on human cells.

1.5.3 $CD34^+$ HSC transplanted mice – the hNOG and RAG-hu models

Given the limitations of SCID-hu and hu-PBL-SCID mice and the fact that no primary immune responses were generated, these models did not fully match the demand for a small HIV animal model. Thus, a model that closely resembles a functional human adaptive immune system including the repopulation and structuring of primary and secondary lymphoid organs was required.

At present, the two recipient mouse models fulfilling these criterions best seem to be:

1) $CD34^+$ HSC transplanted NOD-SCID *Il2rg*$^{-/-}$ mice, independently developed in the laboratories of Mamoru Ito [47] and Leonard Shultz [48] - hereafter referred to as hNOG mice, and
2) $CD34^+$ HSC transplanted BALB/c *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice independently developed in the laboratories of Markus Manz [49] and Hergen Spits [54] - hereafter referred to as RAG-hu mice (Table 1).

Abbreviation	Common Strain Name	Strain nomenclature	Properties	Refs
hNOG	NOD/LtSz-*scid Il2rg*$^{-/-}$	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ	- Long lifespan - Strong reduction in innate immunity - NK cells absent - High level of engraftment of human cells - Develop functional human immune system - Complete absence of *Il2rg* gene	48,51
hNOG	NOD/Shi-*scid Il2rg*$^{-/-}$	NODShi.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/Jic	- Similar to NOD/LtSz-*scid Il2rg*$^{-/-}$ except IL-2R γ-chain is truncated, not absent, and still binds cytokines	47,74
Rag-hu	Balb/c *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$	C.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug}/Jic	- Similar to hNOG mice except RAG-hu are radiation resistant	49
Rag-hu	H2^{d} *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$	Stock (H2^{d})-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Krf}/Brn	- Similar to hNOG mice except RAG-hu are radiation resistant	54

Table 1. Immunodeficient mice with mutations in the IL-2 receptor γ chain appear to be the best model for transplanting human cells and tissues. Adapted from [39].

These mice, created by the injection of $CD34^{+}$ HSC at the neonatal stage, achieve tremendously high levels of engraftment with successive formation of primary and secondary lymphoid organs, and some *in vivo* immune responses making them the favourite candidates for studying HIV pathogenesis. My thesis project entailed the thorough evaluation and establishment of RAG-hu mice as a mouse model for HIV infection (Section 2). In the mean time, several other groups also demonstrated that these models support productive HIV infection closely resembling HIV infection in man. Results will be discussed in section 5.

1.6 Immune activation in HIV pathogenesis

The hallmark of HIV infection is the depletion of $CD4^+$ T cells in peripheral blood, lymphoid organs, and mucosal tissues. The underlying mechanism(s) are not well understood despite almost 30 years of intensive research. Early studies mainly favored direct viral infection and subsequent cytopathic effects as the major cause of $CD4^+$ T-cell loss [4], whereas contemporary studies mainly favour immunopathology and in particular immune activation by HIV as the underlying trigger of progressive immunodeficiency. In this chapter I will discuss the various views about the molecular mechanism(s) resulting in $CD4^+$ T-cell loss with emphasis on immune activation.

1.6.1 Direct viral cytopathogenicity (single-cell killing)

In the eighties, $CD4^+$ T-cell depletion was thought to be mainly due to direct cytopathic effects as a consequence of disruption of the cell membrane as large amounts of virus bud from the surface [75] or as a consequence of intracellular accumulation of viral RNA [76] and unintegrated DNA [77] resulting eventually in host cell apoptosis. Killing of HIV-infected cells by HIV-specific cytotoxic $CD8^+$ T lymphocytes may also contribute to $CD4^+$ T-cell loss. However, this intuitive theory that HIV directly kills $CD4^+$ T cells and that the slow progression to AIDS reflects a long, but eventually lost struggle of the immune system to replace killed cells in its effort to maintain T-cell homeostasis [78,79] is highly unlikely, because only a barely detectable number of cells (0.01-1%) are productively infected in chronic infection [80,81]. The thymus would easily be able to cope with the number of productively infected cells lost. Thus, indirect mechanism(s) are most likely the main determinant for the progressive $CD4^+$ T-cell loss.

1.6.2 Indirect mechanisms - Bystander killing

Uninfected cells may die in several innocent bystander scenarios, including formation of multinucleated giant cells (syncytia formation), immunopathology (immune-mediated

toxicity), autoimmune mechanisms, increased apoptosis, superantigen-mediated anergy, and precursor-cell damage:

Multinucleated giant cells form when the cell membranes of infected cells expressing high levels of gp120 on their surfaces fuse with uninfected $CD4^+$ T cells via conventional HIV receptor complex interactions. These multinucleated giant cells are likely associated with the cytopathic effects of HIV [82,83].

Immunopathology is considered a major determinant of bystander killing. Uninfected cells may be killed when free gp120 binds to their cell surface, giving them the appearance of an infected cell and marking them for antibody-dependent cellular toxicity [84]. Similarly, $CD8^+$ cytotoxic T cells may accidentally destroy uninfected cells that display HIV fragments on their surfaces since they either have digested HIV infected cells or since they have engulfed HIV particles. We would like to emphasize that the role of $CD8^+$ cytotoxic T cells is somewhat controversial: there is circumstantial evidence that the breadth and strength of the $CD8^+$ cytotoxic T cells response is associated with HIV long term non progression [85] indicating that the $CD8^+$ effector cells involved may have a dual role [4].

Autoimmune mechanisms have also been postulated for the killing of innocent bystanders [86]. HIV envelope proteins gp120 and gp41 bear some resemblance to MHC II molecules on $CD4^+$ T cells. Thus, HIV envelope specific antibodies may cross-react with MHC II and, thus, mistakenly damage MHC II^+ cells [86].

Apoptosis occurs to a greater extent in HIV-infected than in non-infected individuals [87]. Of note, HIV proteins such as gp120, Tat, Nef, and Vpu have been shown to induce apoptosis in $CD4^+$ T-cells, even if not infected by HIV [88].

The presence of an HIV-encoded superantigen represents another possible mechanism that explains bystander killing. Superantigens are polyclonal stimulators of antigen-nonspecific $CD4^+$ T lymphocytes. Notably, the Nef protein of HIV is a putative superantigen [89]. Superantigens serve as potent activators of T cells, rendering them more susceptible to HIV infection and ultimately leading to depletion or anergy [89]. Anergy denotes an inactivated state of $CD4^+$ T cells, which become refractory to further *in vitro* stimulation through the activation of their CD3 molecules when their component CD4 molecules reacted with complexes of gp120 antigen and antibody [4].

It has also been observed that HIV infects precursor $CD4^+$ T cells and damages the microenvironment of the bone marrow and the thymus, which probably loose the ability to regenerate the $CD4^+$ T-cell pool.

1.6.3 Role of immune activation in $CD4^+$ T-cell depletion

It is now widely accepted that chronic immune activation drives both viral replication and $CD4^+$ T-cell depletion [78]. Several observations implied a crucial role of chronic immune activation in HIV pathogenesis: $CD4^+$ T cells expressing the activation markers, CD69, CD25, and MHC II as well as $CD8^+$ T cells expressing the activation marker CD38 correlate extremely well with HIV disease progression and $CD4^+$ T cell loss in natural infection [90-92]. Moreover, $CD4^+$ T-cell depletion during the chronic phase is more directly related to the overall activation and turnover of T cells than to the viral replication rate [78,93-95].

Immune activation also facilitates HIV infection of T cells by promoting the upregulation of CCR5 and adhesion molecules and by promoting nuclear-factor (NF)-κB activation [96-98]. This in turn results in increased cytokine production that not only may support HIV replication but also have deleterious consequences. Chronic low-level TNF-α expression, for example, caused profound disturbances in tissue development, especially a diminution of thymic tissue [99] and may be at least partly responsible for the severe weight loss and wasting syndrome seen in advanced stages of HIV infection [100]. The significance of cytokine dysregulation for HIV disease progression is also underscored by polymorphisms that reduce IL-10 expression. Low IL-10 levels have been associated with slower progression to AIDS [101]. Immune activation may also destroy the microachitecture of secondary lymphoid organs such as lymph nodes [102] that will impair the regenerative potential of the immune system [103].

Immune activation seems to be the primary parameter distinguishing pathogenic from nonpathogenic SIV infection in monkeys. SIV-infected rhesus macaques show sustained immune activation [104] and progress to AIDS within a short-time. In contrast, immune activation is absent in natural SIV infection of Sooty mangabeys and African green monkeys, which rarely progress to immunodeficiency despite high levels of viral replication [16]. A cross-sectional study that analyzed more than 100 naturally SIV-infected Sooty mangabeys revealed that $CD4^+$ T-cell levels were not related to the level of viral replication or the length of infection but rather to the set point level of T-cell activation [105].

In acute HIV infection, HIV depletes more than half of the total $CD4^+$ $CCR5^+$ T-cell pool within a few weeks, mainly in the gut-associated lymphoid tissue [106]. This led to the question whether chronic immune activation and T-cell proliferation may simply be a homeo-static response to massive early $CD4^+$ T-cell depletion and not be causally responsible for subsequent $CD4^+$ T-cell depletion and disease progression [103]. This is unlikely because T-cell

activation declines rapidly during antiviral therapy, despite minimal $CD4^+$ T-cell recovery in gut associated lymphoid tissue and long before significant $CD4^+$ T-cell recovery in peripheral blood [103,106,107]. In contrast, homeostatic mechanism aimed at compensating for virus-induced cell loss may be involved in SIV-infected Sooty mangabeys (non-pathogenic infection). Here, induction of antiviral therapy is not followed by a rapid decline in T-cell proliferation [105].

Therapeutic approaches that reduce immune activation may prevent $CD4^+$ T-cell depletion and disease progression. This in turn, necessitates a better understanding of the precise mechanisms of how HIV induces immune activation.

The definitive trigger by which HIV causes T-cell activation is still unclear. T-cell activation might be partly explained by evolutionary changes in Nef function. Nef from nonpathogenic SIV strains mediated downmodulation of the host T-cell receptor and thereby suppressed T-cell activation, whereas Nef from HIV did not [108]. However, this hypothesis remains controversial [109].

Recently, Toll-like Receptors (TLR) have been proposed as potential key players in immune activation in HIV infection [110]. TLRs belong to the family of pattern recognition receptors, are essential in innate immunity in mammals by recognizing conserved patterns of microorganisms (PAMP), are selectively expressed on cells of the immune system [111], and are classified according to the types of PAMPs they recognize (reviewed in [112]). Brenchley et al. proposed circulating microbial products (a TLR4 ligand), likely derived from the gastrointestinal tract upon breakdown of the mucosal barrier, as a cause of HIV-related systemic immune activation [113]. Immune activation correlates with HIV viremia and declines rapidly after initiation of HAART and long before peripheral $CD4^+$ T-cell recovery [114,115]. These observations suggest a direct role for viral replication products in immune activation [116,117]. In this context, HIV ssRNA is likely to contribute directly to immune activation: human TLR7/8 recognize HIV ssRNA [114,118,119], which encodes for multiple TLR7/8 ligands that indeed induced strong immune activation *in vitro* [114].

We thus hypothesized that sustained immune activation through TLR7/8 is a main driver for chronic immune activation and thereby contributes to the progressive immunodeficiency observed in HIV infection. To explore the effects of sustained TLR7 triggering on the molecular and cellular components of the lymphoid system of mice, we used the synthetic compound R848 which triggers TLR7/8 similarly to HIV ssRNA [114,118,120]. Results will be shown and discussed in section 3 of this thesis.

1.7 Signalling through the HIV receptor complex

HIV infects cells through a receptor complex consisting of the CD4 receptor and one of the chemokine receptors CCR5 or CXCR4. Each component of the receptor complex is involved in signal transduction pathways, raising the question of whether HIV exploits them to optimize conditions for viral replication. HIV may use these particular molecules for more than simple attachment to the cell; viral proteins such as gp120 might function as agonist or antagonist of cellular receptors, modulating their downstream signalling pahtways [121]. An understanding of the role of CD4/CCR5 signalling for viral replication might lead to novel targets for anti-HIV therapy.

1.7.1 Natural signalling through the HIV receptor complex

CD4 contains four immunoglobulin-like extracellular domains, a membrane-spanning segment and a short cytoplasmic tail that noncovalently interacts with the lymphocyte-specific protein tyrosine kinase $p56^{lck}$ [122,123]. CD4 primarily acts as an accessory molecule of the T-cell receptor (TCR)/CD3 complex for binding MHC class II molecules [124]. Co-aggregation with the TCR/CD3 complex, cross-linking with antibodies or binding of IL-16 results in phosphorylation of $p56^{lck}$ [125-127] and a cooperative signalling activity, leading to efficient T-cell growth and activation [128-130] (Fig. 5).

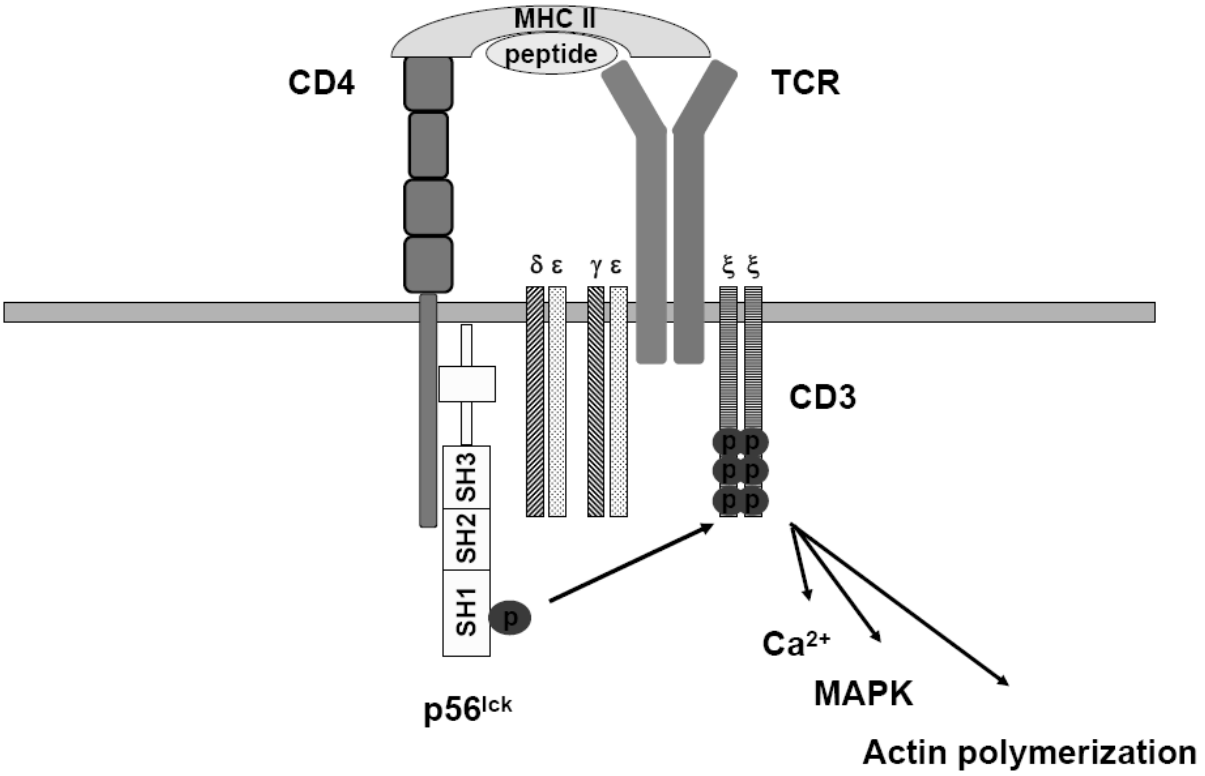

Fig. 5. Scheme of CD4 mediated signalling. Following activation of the TCR/CD3 complex (by binding of e.g., MHC II), the CD4 co-receptor-associated SRC-family protein tyrosine kinase $p56^{lck}$ phosphorylates tyrosine residues in the cytoplasmic domain of the CD3ζ-chains eventually resulting in 1) phospholipase C (PLC)-dependent activation of protein kinase C, which leads to an intracellular calcium flux, 2) RAS-mediated activation of mitogen-activated protein kinases (MAPKs) and 3) actin-dependent cytoskeletal rearrangements.

The two chemokine receptors CCR5 and CXCR4 belong to the superfamily of seven–transmembrane domain receptors [131,132] that signal through heterotrimeric G-proteins upon ligand binding, and are key regulators of leukocyte trafficking to inflamed tissues [133]. Chemokine receptors have several conserved structural features, including a DRY motif in the second intracellular loop, which is involved in G-protein interaction, and structural motifs in the cytoplasmic tail, which are critical for signalling, desensitization, and receptor trafficking [134]. Natural ligands for CCR5 include RANTES, MIP-1α, and MIP-1β. Ligand binding results in a chain of events that alters the concentration of one or more small intracellular signalling molecules [131,132] (Fig. 6).

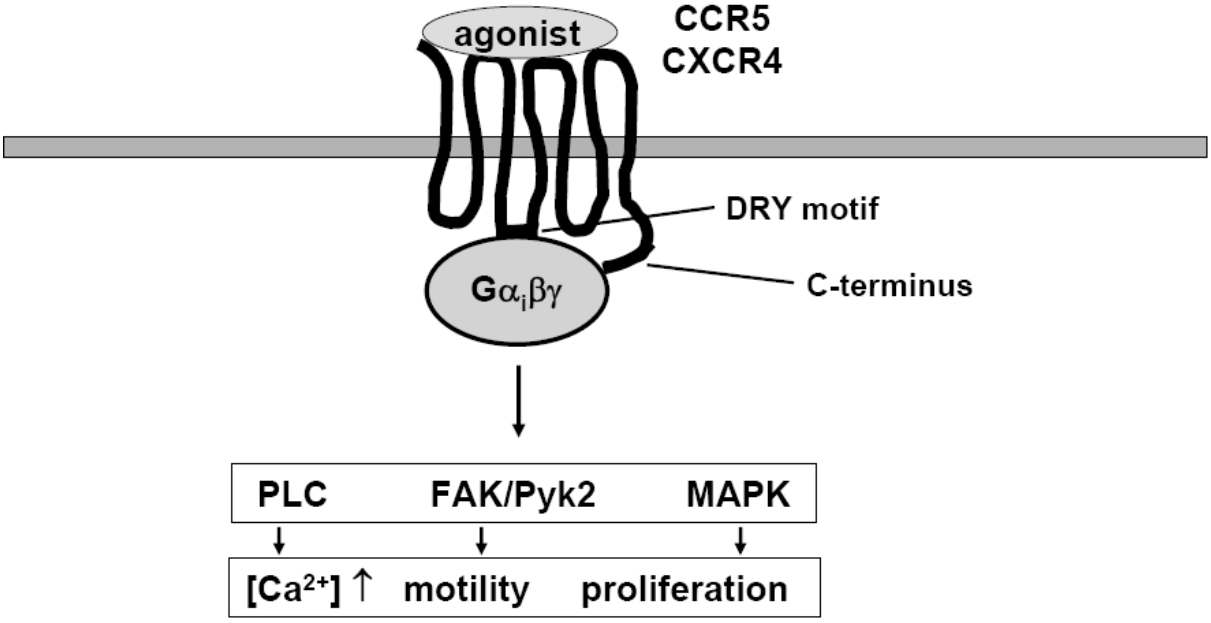

Fig. 6. Scheme of CCR5/CXCR4 G-protein-coupled-receptor (GPCR) mediated signalling. Activation by agonist leads to a conformational change which uncovers G-protein-binding sites. Both the α_i subunit and the $\beta\gamma$ dimers of G proteins can regulate effectors, such as adenylyl cyclase or PLC which result in CCR5-mediated intracellular calcium mobilization. Chemokine-induced activation of T cells via CCR5 leads to activation of the two related focal adhesion kinases FAK and Pyk2, which also have important roles in cell motility including cell spreading and migration. CCR5 ligands activate the three main members of the MAP kinase family (ERK1/2, p38, and SAPK/JNK), which are suggested to be critical for T-cell proliferation and the transcriptional activation of cytokine genes [134].

1.7.2 HIV interactions with the receptor complex induces signalling

As the name implies, R5-tropic HIV strains are dependent on a HIV receptor complex consisting of CD4 and CCR5 [135,136]. Binding of the HIV envelope protein gp120 to CD4 is the initial step in this process [137,138] followed by a subsequent exposure of a cryptic chemokine-like site in gp120, which then binds to CCR5.

This binding of HIV gp120 to CD4 or the chemokine co-receptor also initiates signals (Fig. 7), some of which are specific for HIV, such as activation of non-selective cation channels [139]. Signalling through CD4 or CCR5 does not appear to affect HIV fusion or entry [140-145]. In contrast, post-entry steps of the HIV replication cycle such as reverse transcription, DNA expression, and protein synthesis are affected by CD4-mediated signalling [146 147-151].

After attachment of HIV gp120 to CD4, the CD4 cytoplasmic domain transduces signals [123,152] resulting in aberrant T-cell activation [153], NF-κB activation [141], priming of $CD4^+$ T cells to apoptosis [154], and suppression and deregulation of T-cell responses [153,155].

Similarly, HIV gp120 also signals through chemokine receptors (Fig. 7) and participates in the post-entry stages of the HIV replication cycle [156]. Notably, CCR5 activation affected the early stages of viral replication which required signal transduction through $G\alpha_i$ proteins [157] indicating that activation of intracellular signalling pathway by chemokine co-receptor can have a direct impact on post-entry events that regulate viral replication [121]. Indeed, R5 gp120 binding to CD4 and CCR5 stimulated extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), and p38 [155] which can affect the proliferative capacity and facilitate HIV replication through Activator protein 1 (AP1) and NF-κB-mediated pathways that can enhance the expression of viral genes or numerous cytokines [121]. Moreover, HIV gp120 of either CCR5- or CXCR4-tropic strains can induce calcium flux and chemotaxis and activate cytoplasmic tyrosine kinase Pyk2 [158,159] and the closely related focal adhesion kinase (FAK) [160]. This implies that HIV binding can elicit cytoskeletal rearrangements, which ultimately promote cell polarization and motility. This in turn, could facilitate viral translocation to the nucleus or enhance the recruitment of T cells to sites of viral production and thereby favor viral spreading and dissemination [121].

1.7.3 Limitations of previous studies

Intracellular signals transduced by HIV envelope have been suggested to contribute to the immunopathogenesis of HIV infection by priming cells to become optimal targets for infection or by recruiting uninfected target cells to areas with high concentrations of virus (i.e., lymphoid tissue) [153,158,161]. HIV gp120 has also been shown to induce anergy or prime cells for apoptosis [153,162].

However, it remains unclear whether CD4 negatively [163] or positively [140,141,154,164] regulates HIV replication. Effects of signalling through the chemokine co-receptor CCR5 also are a matter of debate: for example, one study found CCR5-mediated signalling to be dispensable for HIV replication [165], whereas another showed that CCR5 signalling facilitates HIV replication [166].

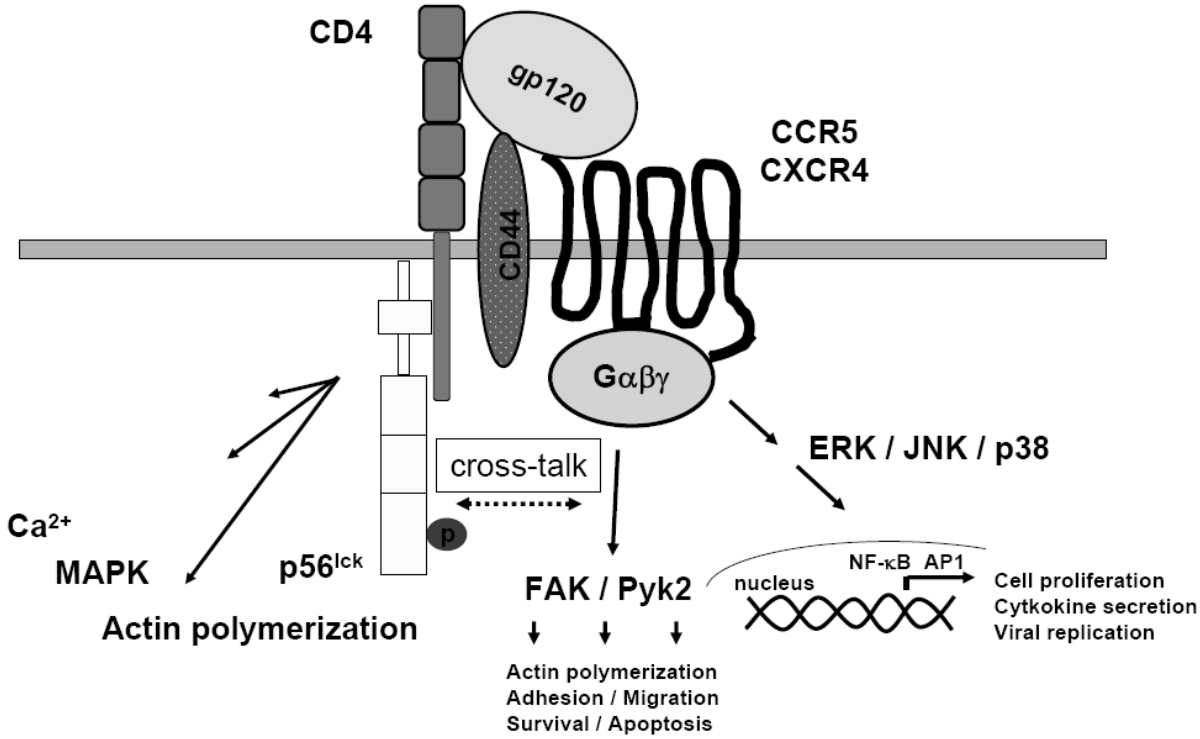

Fig. 7. Interaction of gp120 with the cellular receptor complex induces various signalling events. CD4 stimulation by gp120 induces intracellular calcium flux, MAPK activation, actin polymerization, and cross-talk: an Lck-dependent signal that results in cross-desensitization of CCR5 [167]. CCR5 stimulation activates ERK, JNK, and p38 which promote cell proliferation and transcriptional activity of NF-κB and AP1. The activation of FAK and Pyk2 affects actin polymerization, cell adhesion and migration, and host-cell survival [134]. Cross-talk occurs in both directions. Collectively, all these signalling events might influence viral transmission and replication. However, the role of signalling through CD4 or CCR5 for HIV replication has been a matter of debate.

Moreover, these studies focused on signalling by the components individually. The two receptor (i.e., CD4 and CCR5), however, are physically [168] and functionally [167,169,170] associated and there is cross-talk between the receptors [169,170].

A large body of data has accumulated concerning interactions of the HIV envelope and the viral receptor complex. However, no work has yet examined the motifs of CD4 or CCR5 (or combinations of motifs) that are critical for HIV replication, cell activation, cell apoptosis, or cytokine release. Since HIV entry is a continuous process and cross-talks are known between

the transduction pathways of CD4 and CCR5, they can not be dissected into single components and signalling can not be attributed to an individual event. Therefore, we generated human T-cell lines stably expressing different combinations of either wild-type or mutant CD4 and CCR5. These cell lines were used to study both the overall influence of signalling through the receptor complex on HIV replication and the contribution of each receptor to such effects. Results will be shown and discussed in section 4 of this thesis.

1.8 Aims of this thesis

The aims of this thesis were to study host-HIV interactions *in vitro* and *in vivo*s:

1) To evaluate RAG-hu mice as a model system for HIV infection.

Manz et al. recently established a new human-to-small animal xenotransplantation model (RAG-hu mice) [49]. In brief, transplantation of $CD34^+$ cord blood cells into newborn $Rag2^{-/-}$ $Il2rg^{-/-}$ mice led to *de novo* formation of a human adaptive immune system, (i.e., functional B, T, and myeloid and plasmacytoid dendritic cells). Thus, they have several significant advantages over other available mouse models: de novo T-cell generation from $CD34^+$ cells with a broad T-cell receptor repertoire over at least 6 months, T-cells of naïve and memory phenotype, repopulation of lymphoid organs such as lymph nodes and spleen with cells from the lymphoid and monocytic lineage, generation of primary immune responses. Importantly, RAG-hu mice represent a very straightforward, easily applicable and ethically less problematic model as compared to SCID-hu mice where fetal tissue is transplanted. We examined and thoroughly validated RAG-hu mice for its ability to serve as a small animal HIV model. Read **chapter 2.**

2) To elucidate whether triggering Toll-like receptor (TLR) 7 may be responsible for the chronic immune activation and dysfunction seen in HIV infection.

Chronic immune activation is considered a major contributor to progressive immunodeficiency observed in HIV infection. Indeed, chronic immune activation is superior as prognostic predictor than serial measurements of $CD4^+$ T-cell counts or of HIV RNA. The underlying trigger, however, remains unknown. We hypothesized hat HIV ssRNA itself directly contributes to immune activation and dysfunction by continuously triggering TLR7. We used the synthetic compound R848 which triggers TLR7/8 similarly to HIV ssRNA to explore the effects of sustained TLR7 triggering on the molecular and cellular components of the lymphoid system of mice. Read **chapter 3**.

Building upon the data generated in this project we may extend our explorations in the RAG-hu mouse model. This, however, is beyond the scope of this thesis.

3) **To investigated the impact of signalling through the HIV receptor complex (i.e., CD4 and chemokine receptor CCR5) on HIV replication.**

HIV infects cells through a receptor complex consisting of the CD4 receptor and one of the chemokine receptors CCR5 or CXCR4. Each component of the receptor complex is involved in signal transduction pathways, raising the question whether HIV exploits them to optimize conditions for viral replication. The effects of signalling are a matter of debate probably because previous studies have focused on signalling by the components individually. However, the two receptors are physically associated, which results in reciprocal modulation of their signalling responses. We thus analyzed replication of several CCR5-tropic HIV molecular clones and patient isolates in a panel of cell lines stably expressing different combinations of either wild-type or mutated CD4 and CCR5. Mutations were chosen based on motifs known to be involved in signal transductions. Read **chapter 4**.

2

Mice with a human adaptive immune system (RAG-hu mice) as a potential novel animal model for studying HIV infection

This part contains:

1) Summary
   Published in Baenziger et al. at *Retrovirology* 2006, 3 (Suppl I):S31

2) Disseminated and sustained HIV Infection in $CD34^+$ cord blood cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice.
   Published in Baenziger et al. at *PNAS* 2006, 103 (43):15951-6

3) Discussion: Human T cell development and HIV infection in Human Hemato-Lymphoid System Mice.
   Baenziger et al. at *Current Topics in Microbiology and Immunology* 2008, Vol.324

4) Outlook

2.1 Summary: Disseminated and Sustained HIV-Infection in CD34$^+$ Cord Blood Cell Transplanted Rag2$^{-/-}\gamma_c^{-/-}$ Mice

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Background

Due to species selectivity, HIV research is largely restricted to *in vitro* or clinical studies, both limited in their ability to rapidly assess new strategies to fight the virus. We evaluated a novel mouse model that is based on the transplantation of human cord blood CD34$^+$cells into immunodeficient Rag2$^{-/-}\gamma_c^{-/-}$ newborn mice for studying HIV.

Results

Reconstituted mice (CD45$^+$ cell engraftment: 29 ± 18%) were permissive to HIV with up to 2 × 10^6 copies/ml 2–6 weeks after infection. Thereafter, viremia stabilized at lower levels for up to 4 months. A marked CD4$^+$ cell depletion occurred in all mice infected with X4- strains simultaneously with the initial rise of plasma HIV RNA while this was not the case in R5-infected mice. Spleens and lymph nodes of mice infected with either R5- or X4- strains contained p24$^+$ cells. In thymi, however, p24$^+$ cells were detected rather exclusively following infection with X4- strain, consistent with the expression of CXCR4 but not CCR5 on human CD4$^+$ thymocytes. Similarly as in humans, HIV-infected macrophages were rare.

Conclusion

Rag2$^{-/-}\gamma_c^{-/-}$ mice transplanted with human CD34$^+$ cells develop long-term, high-titer, and lymphoid organ disseminated infection irrespective of co-receptor selectivity of HIV strain, closely resembling HIV infection in man. In particular, by using HIV strains with distinct co-receptor selectivity, we clearly illustrate the higher cytopathic potential of X4- strains as compared to R5- strains. This straightforward to generate and cost-effective *in vivo* model should be valuable to study virus-induced pathology, and to rapidly evaluate new approaches aiming to prevent or treat HIV infection.

2.2 Disseminated and Sustained HIV-Infection in CD34$^+$ Cord Blood Cell Transplanted Rag2$^{-/-}\gamma_c^{-/-}$ Mice

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Due to species selectivity, human immunodeficiency virus (HIV) research is largely restricted to *in vitro* or clinical studies, both limited in their ability to rapidly assess new strategies to fight the virus. To prospectively study some aspects of HIV *in vivo*, immunodeficient mice, transplanted with either human peripheral blood leukocytes or with human fetal tissues, have been developed. Although these are susceptible to HIV infection, xeno-reactivity and short infection spans, resource and ethical constraints, as well as biased HIV co-receptor tropic strain infection, pose substantial problems in their use. Rag2$^{-/-}\gamma_c^{-/-}$ mice, transplanted as newborns with human CD34$^+$ cells were recently shown to develop human B, T, and dendritic cells, constituting lymphoid organs *in situ*. We here tested these mice as a new model system for HIV-1 infection. HIV RNA levels peaked to up to 2x10^6 copies/ml plasma early after infection, and viremia was observed for up to 190 days, the longest time followed. A marked relative CD4$^+$ T cell depletion in peripheral blood occurred in CXCR4-tropic strain infected mice, while this was less pronounced in CCR5-tropic strain infected animals. Thymus infection was almost exclusively observed in CXCR4-tropic strain infected mice, while spleen and lymph node HIV infection occurred irrespective of co-receptor selectivity, consistent with respective co-receptor expression on human CD4$^+$ T cells. Thus, this straight-forward to generate and cost-effective novel *in vivo* model closely resembles HIV infection in man, and therefore should be valuable to study virus-induced pathology, and to rapidly evaluate new approaches aiming to prevent or treat HIV infection.

2.2.1 Introduction

Since the beginning of the HIV pandemic, research has been hampered due to the lack of assessable animal models that mirror infection in man. HIV is a human-specific virus, and consequently laboratory rodents as mice or rats are not susceptible to infection [9]. Although non-human primates as chimpanzees can be infected, they do not develop HIV-associated immunodeficiency [12,13], while sooty mangabeys, rhesus macaques, and baboons are only susceptible to HIV related simian-immunodeficiency virus [14]. Therefore, HIV research in non-human primates, although of importance, remains restricted by biological as well as by ethical and financial constraints [171]. Efforts to genetically engineer rodents to become HIV targets (e.g., artificial expression of human CD4, CCR5, or CXCR4) have largely failed, because, even if infection *in vitro* was achieved, HIV replication *in vivo* was limited or absent [9,172,173]. Thus, substitute xeno-chimeric models have been developed by transplanting immunodeficient mice with either human peripheral blood leukocytes (hu-PBL-SCID) [68,174], or pieces of human fetal tissues containing hematopoietic cells (SCID-hu) [56,175]. Both hu-PBL-SCID and SCID-hu mice sustain HIV infection and replication *in vivo*. However, in hu-PBL-SCID mice xeno-reactivity and successive loss of human leukocytes limit infection to a relatively short time frame, and, possibly driven by activation-induced CCR5 expression on human cells, infection is skewed towards HIV strains with the respective co-receptor tropism [72,176]. In SCID-hu mice, due to the transfer of human hematopoietic stem cell containing tissue, HIV infection can be observed for extended times; however, availability of transplantable human fetal organs is restricted for practical and ethical reasons, and HIV pathology in these mice is mainly limited to the tissue implants, with naïve T cells being preferentially susceptible to CXCR4-tropic strain infection [9,59,177]. Given these limitations and the fact that no primary immune responses were generated, hu-PBL-SCID and SCID-hu mice did not fully match the demand for a small animal model that closely mirrors infection in humans.

Recently, we found that injection of human cord blood $CD34^+$ cells into newborn $Rag2^{-/-}\gamma_c^{-/-}$ mice leads to development of human T-, B- and dendritic cells, successive formation of primary and secondary lymphoid organs, and some *in vivo* immune responses [49,178]. We here evaluated these mice as a new model system for both CXCR4- and CCR5-tropic HIV-1 infection.

2.2.2 Results

CXCR4 and CCR5 expression on *de novo* generated human thymocytes and $CD4^+$ T cells in $Rag2^{-/-}\gamma_c^{-/-}$ mice.

Depending on the use of chemokine receptors in combination with CD4 for cellular entry, HIV has been classified into CXCR4- or CCR5- tropic strains [135,136,179]. In humans, $CD4^+$ thymocytes and $CD4^+$ T cells broadly express CXCR4, while CCR5 expression is restricted to a fraction of mainly $CD4^+$ T memory cells [180]. Similarly, most of thymic and peripheral $CD4^+$ T cells in $CD34^+$ cord blood cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice expressed CXCR4, while CCR5 was found only on a fraction of lymphoid organ $CD4^+$ T cells that almost exclusively displayed a memory phenotype as determined by CD45RO expression (Fig. 1 and data not shown). Thus, *de novo* generated human $CD4^+$ thymocytes and T cells in $CD34^+$ cord blood cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice closely resemble chemokine receptor expression patterns observed in humans, and therefore should be valid targets for HIV strains with respective co-receptor selectivity.

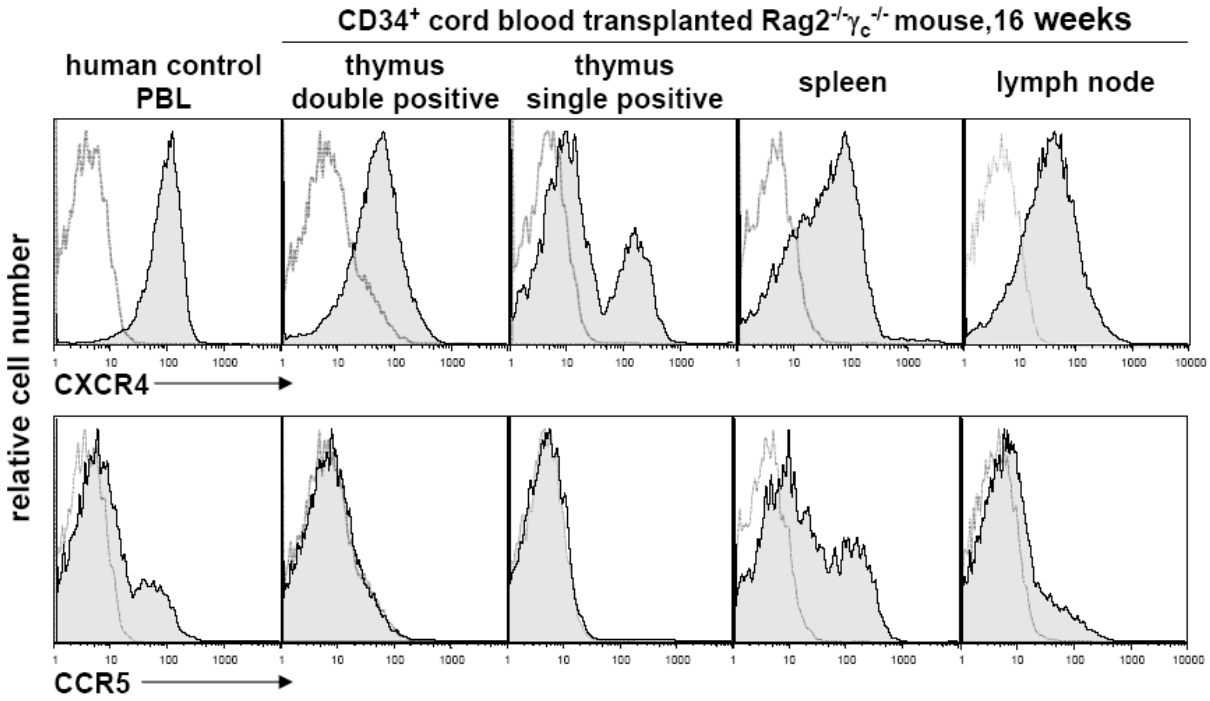

Fig. 1. CCR5 and CXCR4 expression on human $CD4^+$ cells in $CD34^+$ transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice resemble expression patterns in man. Histograms depict representative receptor expressions (closed histograms) and respective isotype controls (open histograms) on $CD4^+$ gated control human PBL and cells isolated from tissue of a mouse at 16 weeks after birth and transplantation of $CD34^+$ cells.

Long-term and high-titer CCR5- and CXCR4-tropic HIV infection in human CD34$^+$ cord blood cell transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice.

CD34$^+$ cord blood cell transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice with a mean human peripheral blood CD45$^+$ and CD4$^+$ cell chimerism of 29.4±18.2% and 2.7±3.0%, respectively (detailed analysis of human cell engraftment in representative animals are given in supplementary Table 1), were infected i.p. with either CCR5-tropic YU-2 (n=15), or CXCR4-tropic NL4-3 (n=19) HIV-strains at 10-28 (mean 16.4±6.7) weeks of age. Plasma levels of viral RNA were measured at successive time-points. Independent of viral strains used, HIV RNA levels peaked two to six weeks after infection, with up to about $2x10^6$ copies/ml plasma, while thereafter viremia mostly stabilized at lower levels, and was maintained for up to 190 (YU-2) and 120 (NL4-3) days, the longest time followed (Fig. 2A). No HIV RNA was detectable at four weeks post-injection in two YU-2 and five NL4-3 receiving animals. However, upon NL4-3 re-infection of four of the latter ones, all four became HIV RNA positive.

In a subgroup of both CCR5- and CXCR4-tropic strain infected mice (n=4 each), we measured HIV RNA as well as relative CD4$^+$ and CD8$^+$ cell counts in peripheral blood over time: partial CD4$^+$ T cell depletion occurred in three out of four mice infected with CCR5-tropic strains beyond 125 days of infection, while all CXCR4-tropic strain infected animals showed a more pronounced relative peripheral blood CD4$^+$ T cell depletion already early in infection, i.e., beyond day 25, following the initial rise of plasma HIV RNA (Fig. 2B).

HIV strains recovered from either YU-2 or NL4-3 infected chimeric Rag2$^{-/-}\gamma_c^{-/-}$ mice were fully functional as co-cultured mouse spleen cells propagated infection in primary human peripheral blood leukocytes (PBL) *in vitro* (Fig. 3). Together these findings indicate that CD34$^+$ cord blood cell transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice have an overall high susceptibility to both CXCR4- and CCR5-tropic HIV, and develop high and sustained viral titers, comparable to titers found in HIV infected individuals.

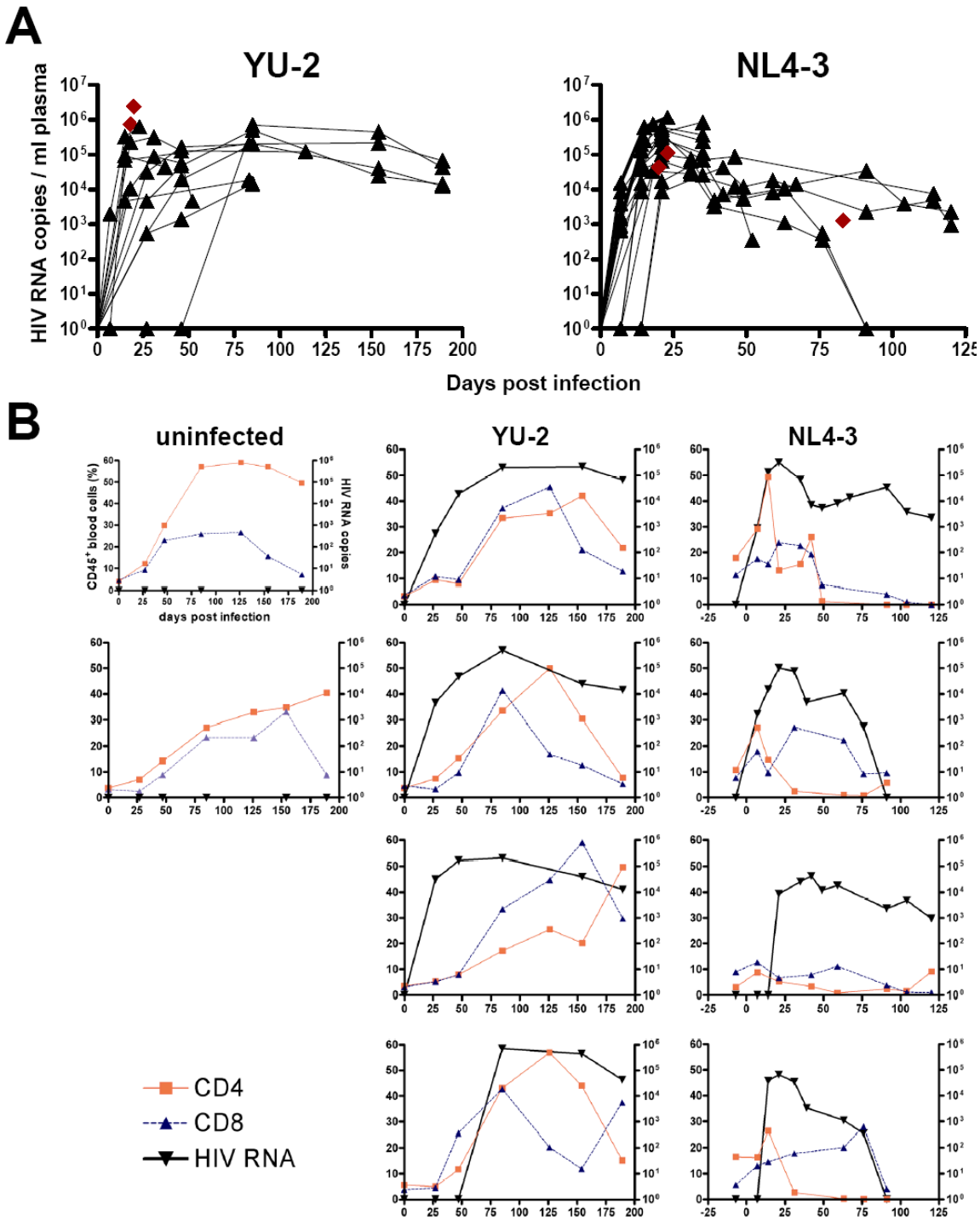

Fig. 2. Long-term and high-titer CCR5- and CXCR4-tropic HIV infection in human CD34$^+$ cord blood cell transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice. A) Quantitative HIV RNA plasma levels in animals successfully infected with CCR5-tropic (YU-2; n=13) and CXCR4-tropic (NL4-3; n=18) HIV strains. Black triangles and connector lines indicate sequential analysis of single mice, brown diamonds indicate single time-point analyzed mice. B) Graphs depict quantitative HIV RNA plasma levels (copies/ml plasma; right y-axis) and relative CD4$^+$ and CD8$^+$ T cell levels (% of human CD45$^+$ blood cells; left y-axis) in individual mice over time (uninfected n=2, YU-2 and NL4-3 infected n=4 each), showing more pronounced CD4$^+$ T cell depletion in CXCR4-tropic infected animals.

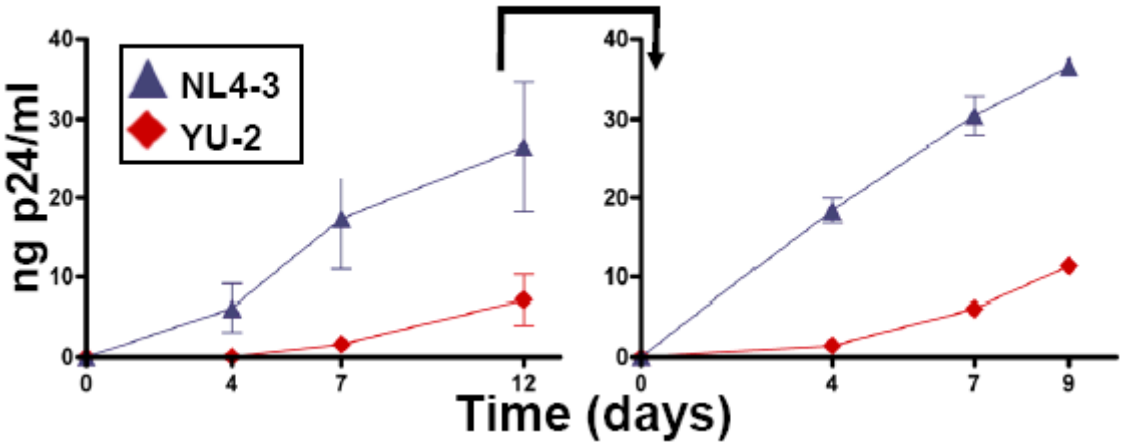

Fig. 3. CXCR4- and CCR5-tropic HIV infected, chimeric Rag2$^{-/-}\gamma_c^{-/-}$ mice passaged HIV propagates infection to primary human PBL *in vitro*. Left graph shows p24 levels in supernatants of HIV-infected mice splenocytes and PHA-activated human PBL co-cultures. Right graph shows p24 levels in PHA-activated PBL cultures infected with transferred supernatants of primary cultures (left), proving integrity of virions released from infected mouse derived splenocytes (representative experiment of 2; blue triangle NL4-3, red daimond YU-2).

HIV spreading in lymphoid organs of human CD34$^+$ cell transplanted Rag2$^{-/-}\gamma c^{-/-}$ mice resembles HIV infection in humans.

To visualize the major cell types productively infected with HIV in lymphoid organs, serial sections were taken and stained with anti-HIV p24 capsid antigen and anti-human CD3, revealing that, as expected, most of infected cells were human T cells (Fig. 4A). Although CD68$^+$ macrophages, CD11c$^+$ dendritic cells, and CD14$^+$ monocytic cells were generated from human CD34$^+$ cells in mice (Fig. 4B, C, D and Table 1,) [49], p24 expressing, and thus productively HIV infected non-T cells such as CD68$^+$ macrophages were only occasionally detected (Fig. 4C, D), suggesting that non-T cells are a minor source of HIV in this model.

From 18 days post-infection, spleens and lymph nodes of both NL4-3 and YU-2 infected animals contained p24$^+$ cells. In thymi, however, p24 expression was detected in NL4-3, but only infrequently or not at all in YU-2 infected animals, consistent with the expression of CXCR4 but not CCR5 on human CD4$^+$ thymocytes (Fig. 1, Fig. 5).

In both CXCR4- and CCR5-tropic HIV infected mice, p24$^+$ multinucleated giant cells were formed in lymph node and spleen sections (Fig. 4C), a phenomenon previously reported in brain and lymphoid tissues of HIV-infected individuals.

Thus overall, the here observed HIV dissemination in lymphoid organs of human CD34$^+$ cell transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice closely resembles HIV infection in humans.

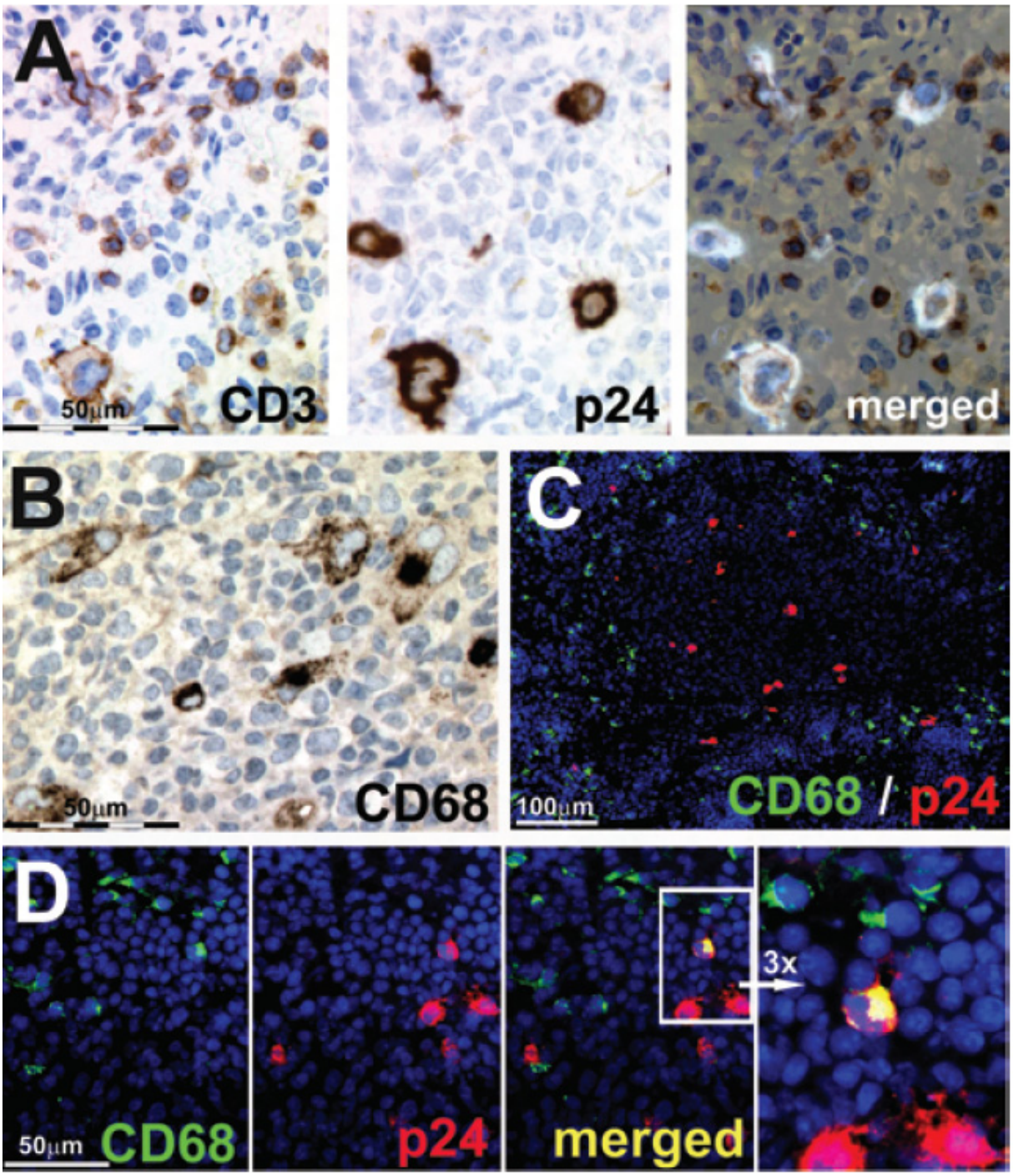

Fig. 4. HIV p24^{+} cells are mostly human CD3^{+} cells and only occasionally non-T cells such as CD68^{+} macrophages. A) Histologies show consecutive spleen sections (paraffin, 1µm) stained with antibodies against human CD3 and p24, and respective merged presentation of both in a YU-2 infected animal 18 days post infection. B) Anti human CD68 staining on paraffin embedded material; C) merged anti CD68 (green), HIV-p24 (red), and DAPI (blue) staining of cryo-embedded spleen (6µm sections), showing that p24^{+} cells mainly localize in white pulp areas (darker area, see also Fig. 4B), whereas CD68^{+} cells mostly localize at adjacent margins and red pulp areas. D) Consecutive spleen cryosection staining and respective merged presentation showing a rare CD68 and p24 double positive cell (yellow). Right: 3x enlargement of area with double positive cell. B, C, D) representative spleen sections from a YU-2 infected animal 23 days post infection.

Table 1: Distribution of human cells in CD34+ cord blood cell transplanted mice

	not infected				YU-2 infected				NL4-3 infected			
Days post infection					**15**	**15**	**22**	**23**	**15**	**15**	**22**	**23**
Blood												
● CD45	75.9	37.2	12.4	5.3	18.7	4.8	3.5	29.3	5.3	4.9	5.2	43.1
* CD4	52.2	43.1	12.0	21.1	7.1	5.1	8.6	12.7	14.7	11.7	36.0	38.3
* CD8	31.2	44.2	8.2	8.3	6.5	3.4	2.9	9.5	8.1	5.2	20.9	46.7
* CD19	10.4	8.9	n.a.	n.a.	n.a.	n.a.	n.a.	62.6	n.a.	n.a.	n.a.	4.6
Bone marrow												
● CD45	89.9	45.7	20.2	4.9	52.5	38.3	22.1	22.0	8.4	34.4	1.4	82.0
* CD4	32.2	15.3	2.0	8.2	7.9	5.1	6.4	4.4	3.9	4.6	32.4	47.8
* CD8	34.7	5.6	0.7	1.3	0.2	0.3	3.2	1.7	0.6	0.1	27.0	25.1
* CD19	25.5	52.4	n.a.	n.a.	n.a.	n.a.	n.a.	59.1	n.a.	n.a.	n.a.	9.7
* CD34	n.a.	1.2	7.4	2.9	16.0	12.3	12.4	4.0	2.6	11.1	1.3	10.4
* CD123	n.a.	7.7	1.0	1.1	2.6	1.2	1.4	7.8	1.5	1.4	2.6	1.3
* CD11c	n.a.	n.a.	n.a.	2.9	8.6	4.4	8.1	n.a.	3.2	5.1	27.8	n.a.
Spleen												
● CD45	93.2	55.8	28.0	24.0	27.6	17.1	22.5	26.0	22.2	19.0	6.9	10.1
* CD4	41.3	25.6	4.8	7.1	4.8	1.4	1.7	9.0	5.5	3.5	12.0	33.6
* CD8	18.7	29.9	4.8	5.3	4.0	1.0	1.8	5.5	4.7	2.4	21.5	50.2
* CD19	32.7	36.9	n.a.	n.a.	n.a.	n.a.	n.a.	72.7	n.a.	n.a.	n.a.	9.6
* CD14	0.2	2.5	n.a.	n.a.	n.a.	n.a.	n.a.	5.4	n.a.	n.a.	n.a.	1.0
* CD123	<0.1	<0.1	0.8	0.3	0.5	0.8	0.4	1.0	0.5	0.6	1.6	<0.1
* CD11c	13.4	13.6	1.4	1.6	3.6	0.8	1.1	8.0	1.6	1.1	6.7	11.1
Lymph node												
● CD45	94.8	88.7	81.8	74.8	91.0	60.5	71.7	86.6	86.8	74.4	66.6	89.7
* CD4	75.5	39.5	30.3	31.2	27.7	42.1	36.3	29.3	44.1	38.6	7.3	6.3
* CD8	n.a.	n.a.	33.3	32.8	33.7	30.9	14.4	n.a.	24.7	28.8	43.7	n.a.
* CD123	1.5	3.2	n.a.	n.a.	n.a.	n.a.	n.a.	0.2	n.a.	n.a.	n.a.	7.1
Thymus												
● CD45	92.3	82.5	95.7	94.9	94.1	98.2	93.6	98.1	99.0	97.3	94.4	87.4
* CD4 single pos.	n.a.	n.a.	21.0	44.2	26.0	13.9	42.1	n.a.	33.3	11.5	44.7	n.a.
* CD8 single pos.	n.a.	n.a.	18.7	14.3	31.4	6.8	13.1	n.a.	4.1	8.4	32.4	n.a.
* double neg.	n.a.	n.a.	16.1	9.3	19.9	5.5	6.6	n.a.	3.7	10.4	8.8	n.a.
* double pos.	n.a.	n.a.	44.2	32.2	22.7	73.8	38.2	n.a.	58.9	69.7	14.1	n.a.
Liver												
● CD45	n.a.	n.a.	67.1	36.6	77.6	33.7	26.5	n.a.	48.2	41.5	31.4	n.a.
* CD4	n.a.	n.a.	30.2	28.7	35.4	16.9	25.2	n.a.	22.8	27.0	17.9	n.a.
* CD8	n.a.	n.a.	9.8	9.3	8.1	3.1	3.1	n.a.	5.9	6.1	30.9	n.a.
* CD123	n.a.	n.a.	4.8	3.6	3.5	5.8	8.0	n.a.	2.3	5.3	0.7	n.a.
* CD11c	n.a.	n.a.	8.6	11.1	38.9	9.3	24.9	n.a.	11.6	15.5	5.0	n.a.

● Percentage (%) human CD45 positive cell of all viable mononucleated cells

* Percentage (%) respective cells of human CD45 positive cells

n.a.: not assessed. Distribution of human cell engraftmen in respresentative mice (mean age 16.6 weeks, range 14.6-21.9 weeks) as assessed by flowcytometry.

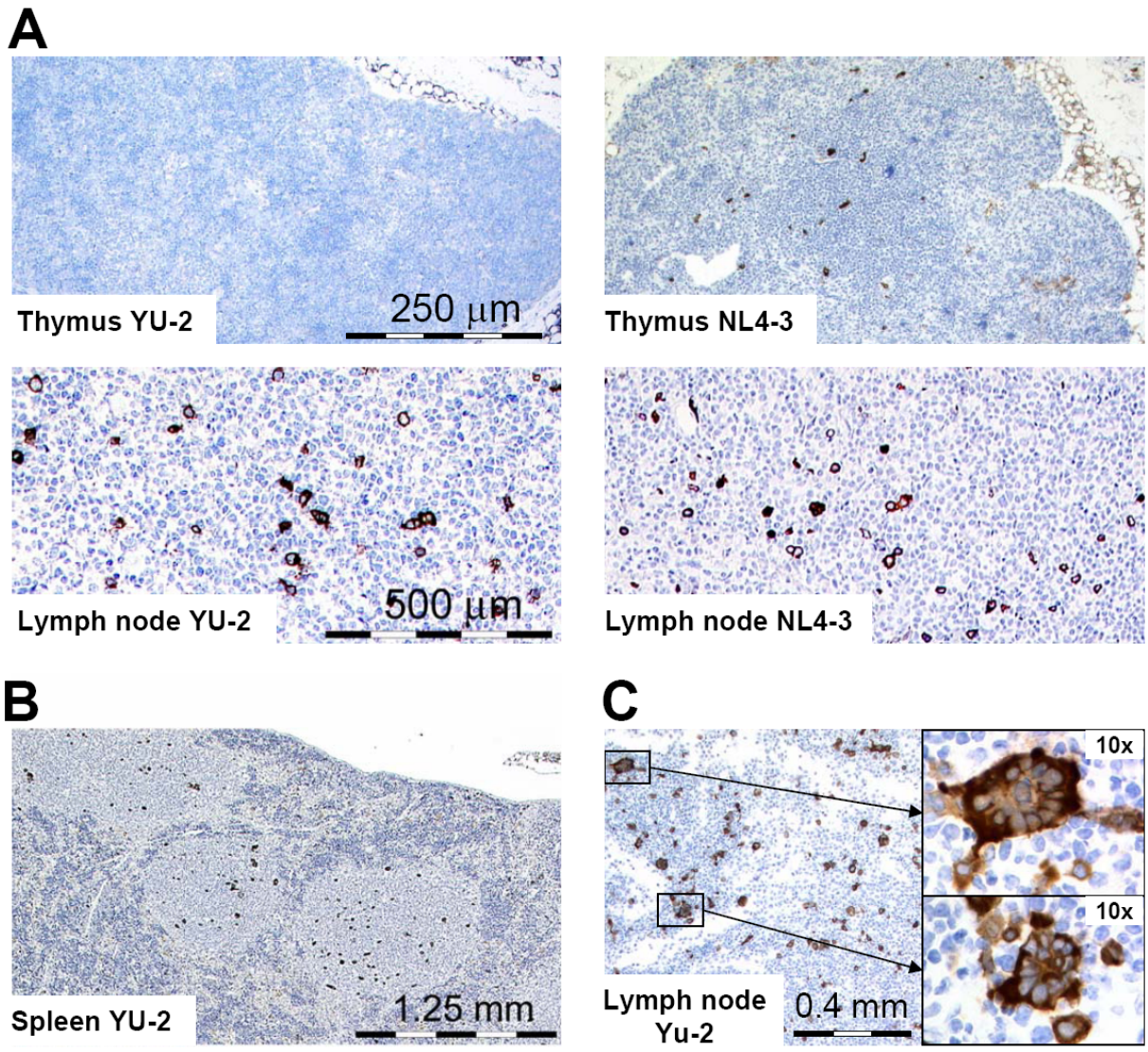

Fig. 5. HIV spreading in lymphoid organs of human CD34^{+} cell transplanted Rag2$^{-/-}\gamma c^{-/-}$ mice resembles HIV infection in humans. A) Representative p24 stained thymus and lymph node sections of YU-2 and NL4-3 infected mice, analyzed at 37 and 23 days post-infection, respectively. No or very rare p24 staining is observed in thymi of R5 tropic YU-2 infected animals. B) Representative tissue section of spleen and C) lymph node of a YU-2 infected animal at 52 days post infection, showing multinucleated p24^{+} giant-cells (enlarged inserts in C).

Human CD34^{+} cell transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice mount no or very limited B cell responses to HIV-infection.

To determine human B cell responses in Rag2$^{-/-}\gamma_c^{-/-}$ mice beyond three weeks of infection, plasma samples of n=23 were tested for total human IgG levels, and n=25 were analyzed for HIV specific IgM and IgG by western blot. As expected from our previous observations [49], total IgG levels accounted for a mean of 0.136 g/l (range 0.0295-0.5 g/l), an amount about 80 times less than found in healthy human adults. Only one animal infected with YU-2 at 23

weeks of age (11% human $CD45^+$ cells in peripheral blood), and analyzed at 42 days post-infection, produced a detectable IgG, but no measurable IgM response against p34, gp41 (weak), p52, p58, and gp160 (Fig. 6).

We also performed a limited analysis of T cell responses: spleen cells of each two NL4-3 and YU-2 infected animals sacrificed at 52 to 115 days post infection were loaded *in vitro* with HIV specific peptides, covering a broad range of HLA types, followed by flow-cytometric measurement of intracellular IFN-γ production. However, no relevant IFN-γ production could be detected, suggesting a lack of, or below detection limit T cell response.

Together these results indicate that HIV infected human $CD34^+$ cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice mount no substantial or only very occasional B cell, and likewise insufficient T cell responses.

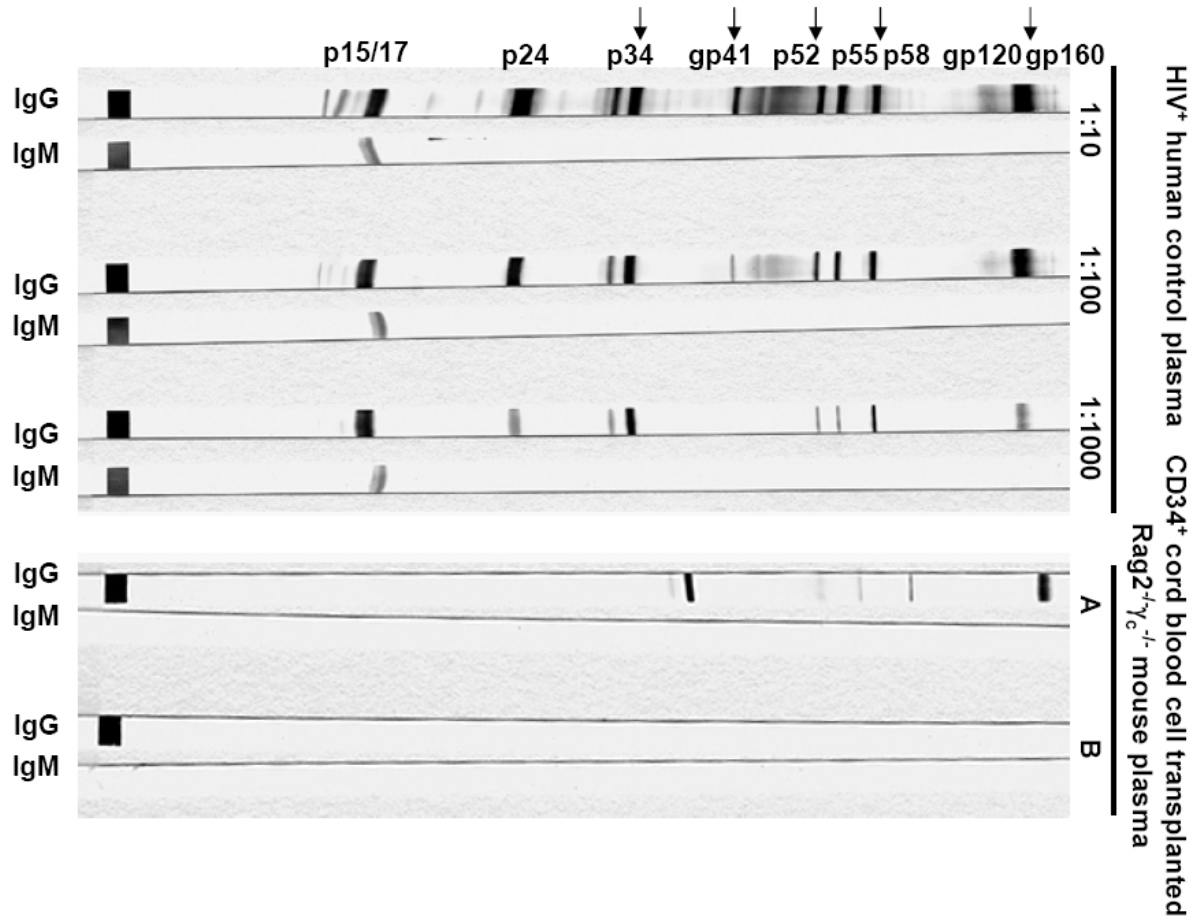

Fig. 6. Humoral immune response in HIV infected human $CD34^+$ cell transplanted $Rag2^{-/-}\gamma c^{-/-}$ mice. Figure depicts western blot analysis of HIV specific IgG and IgM responses. Upper panel: three step dilution of plasma from a HIV infected patient (no antiviral therapy) showing full IgG sero-conversion. Lower panel: undiluted plasma of animal showing a measurable IgG response against p34, gp41, p52, p58, and gp160 (A), and undiluted plasma of an animal showing no response (B).

2.2.3 Discussion

To investigate infectious agents prospectively, suitable *in vivo* laboratory models are needed. This poses a problem in research involving human specific pathogens as HIV. To this end, xeno-chimeric models have been developed by transplanting immunodeficient mice with cellular targets of HIV, i.e., either human PBL [68,174], or pieces of human fetal tissues as liver and thymus, containing hematopoietic cells (SCID-hu) [175]: While suitable to study some aspects of HIV *in vivo*, both models are limited by systematic issues [9]. Human PBL transplanted into SCID mice (hu-PBL-SCID) are activated within the xenogeneic environment, T cells become successively anergic, and, due to lack of both continuing hematopoiesis as well as appropriate hu-PBL maintenance, the xeno-graft is non-functional within several weeks [9,174]. In contrast, mice transplanted with human fetal liver and thymus (SCID-hu) *de novo* generate and maintain human cells, especially T cells, within the human thymus graft [56]. However, SCID-hu mice are laborious and costly to generate, and availability of transplantable human fetal organs is restricted for practical and ethical reasons, limiting broader use of these mice in laboratories. Recently, major advances in generating xenogeneic-mouse models that continuously produce T cells and all other major cell types of the human adaptive immune system in respective mouse organs from human hematopoietic stem and progenitor cells, were achieved (reviewed in: [40,41,181]). We demonstrated that newborn human cord blood $CD34^+$ cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice develop *de novo* human T-, B- and dendritic cells, form primary and secondary lymphoid organs *in situ*, and mount some immune responses upon tetanus toxoid vaccination or infection with Epstein Barr virus (EBV) [49]. Similar results were obtained by transplanting NOD/SCID$\gamma_c^{-/-}$ mice with either human cord blood or mobilized peripheral blood $CD34^+$ cells [48,51,74,182].

We here establish cord blood $CD34^+$ cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice as a new tool to study HIV infection and pathogenesis *in vivo*. In this model system, CXCR4 and CCR5 expression on *in vivo* generated human $CD4^+$ T cells in respective lymphoid organs closely resembles HIV co-receptor expression in humans (Fig. 1) [180]. Accordingly, efficient *in vivo* infection of human cells with both CXCR4-tropic (NL4-3) as well as CCR5-tropic (YU-2) HIV stains, was reliably achieved (Fig. 3, 4, 5), leading to replication of fully functional HIV (Fig. 2). While CXCR4-tropic stains infected all lymphoid organs, CCR5-tropic stain infection was largely restricted to extra-thymic tissues (Fig. 5). Both viral strains led to long-term, high-titer infection with an initial viremic peak of up to $2x10^6$ HIV RNA copies/ml plasma, followed by

a chronic phase with somewhat lower RNA levels for up to 190 days, the longest time followed (Fig. 2).

In both CXCR4- and CCR5-tropic strain infected animals productively HIV-infected cells, i.e., $p24^+$ cells, were mostly $CD3^+$, and only occasionally non T cells such as $CD68^+$ macrophages (Fig. 4, and data not shown). These findings are reminiscent of data acquired from *ex vivo* isolated lymphoid tissue of HIV-infected individuals, where productive macrophage infection by both CXCR4- and CCR5-tropic HIV strains is very infrequently observed [80,183,184] and likely only increases in end-stage disease with occurrence of opportunistic infections [185].

Irrespective of co-receptor selectivity, HIV-infected, multinucleated giant cells were formed (Figure 5C), a phenomenon previously observed in brain and lymphoid tissues of HIV-infected individuals, likely associated with high viral replication, spreading infection, and $CD4^+$ T cell loss [83,186].

Together, these findings are clearly distinct from previous observations on HIV infection in hu-PBL SCID or SCID-hu mice: although both CXCR4- and CCR5-tropic viruses infect hu-PBL-SCID mice, CCR5-tropic viruses are more aggressive, likely due to xenogeneic activation and CCR5 up-regulation of transferred mature T cells, and, with consecutive graft failure, infection is limited to few weeks; in contrast, SCID-hu mice develop few human CCR5 carrying cells, and CXCR4-tropic HIV replication is limited rather exclusively to the fetal tissue grafts; furthermore, in both SCID models by virtue of their nature, no HIV dissemination to respective lymphoid organs occurs.

In man, CCR5-tropic HIV strains are primarily transmitted and dominate infection over extended times. In about half of late stage HIV patients, CXCR4-tropic strains emerge, either as cause or consequence of accelerated immunodeficiency, a matter still under debate [187]. Previously, it was demonstrated that CXCR4-tropic HIV strains lead to pronounced cell depletion in human fetal thymus grafts in SCID-hu mice [177], and replicated more efficiently in primary human thymocytes *in vitro* [188]. Interestingly, CXCR4-tropic HIV infection in $CD34^+$ cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice, lead to more rapid blood $CD4^+$ cell loss than CCR5-tropic infection (Fig. 2). Thus, these findings rather suggest a causative than secondary role of CXCR4 virus in accelerated immunodeficiency, possibly in part by destruction of emerging thymocytes [187,189].

An ultimate goal in the use of substitute xenogeneic small animal models is the generation of robust human primary adaptive immune responses in order to rapidly test potential new

vaccine candidates, a matter unfortunately not met so far in human hematopoietic stem and progenitor cell transplanted animals [9,40,41]. However, some limited evidence for primary immune responses in the xenogeneic setting were reported: SCID-hu mice were resistant to opportunistic infections, suggesting some direct or indirect immune function of the human grafts [56]; we have demonstrated that human $CD34^+$ cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice generate some low-level specific IgG responses to tetanus toxoid upon repeated vaccination beyond 12 weeks of age, and, following EBV infection, some mice showed inverted CD4:CD8 ratios, and CD8 T cells proliferated *in vitro* when stimulated with autologous EBV-transformed target cells [49]; furthermore, ovalbumin specific human IgM and IgG responses were observed in NOD/SCID$\gamma_c^{-/-}$ mice [51]. While in the here presented data all tested HIV infected $Rag2^{-/-}\gamma_c^{-/-}$ mice produced human IgG at levels on average 80 fold lower than healthy human adults, only one out of 25 mounted a detectable HIV-specific IgG response (Fig. 6). Similarly, although based on limited data, we did not detect HIV-specific T cell responses, as determined by IFN-γ detection upon *in vitro* re-stimulation. Low or absent immune responses to HIV might in part be due to preferential destruction of virus reactive cells by HIV [190], however, in this setting the more likely explanation might be inefficient MHC selection of human T cells in the mouse thymus, and possible lack of some cross-reactive cytokines and chemokines in the xenogeneic environment [40,41,49,178]. Thus, generating primary HIV-specific immune responses remains a challenge that might be solved by adding human MHC, cytokines, chemokines, or stromal cell compounds to the recipient mouse background, all efforts currently under way.

In summary, the here presented data establishes newborn human $CD34^+$ cell transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice [49] as a new tool to study HIV infection and pathogenesis *in vivo*. Upon CCR5-tropic or CXCR4-tropic HIV challenge these mice develop long-term, high-titer, and lymphoid organ disseminated infection, closely resembling HIV infection in humans. This straight-forward to generate, cost-effective, ethically unproblematic, and easy to monitor new *in vivo* model should thus be valuable to study virus-induced pathology, as well as pharmacologic or genetic approaches aiming to prevent or treat HIV infection.

2.2.4 Materials and Methods

Cord blood samples: Human cord blood was obtained with written parental informed consent from healthy full-term newborns with approval of the local ethical board. $CD34^+$ cells were enriched using immunomagnetic beads (Miltenyi Biotec) as described [49]. Cells were either frozen or transplanted immediately. Animals used in this study received 50,000-600,000 (mean 227,500 ± 140,000) $CD34^+$ selected cells.

Mice: Human $CD34^+$ cell reconstituted mice were generated as described in accordance with the guidelines of the Institute for Research in Biomedicine (IRB), Bellinzona, animal facility [49]. $Rag2^{-/-}\gamma_c^{-/-}$ mice were originally kindly provided by M. Ito, Kawasaki, Japan.

HIV-1 infection: Viral stocks were obtained by calcium phosphate transfection (Promega) of 293T cells with pNL4-3 or pYU-2. pYU-2 and pNL4-3 were obtained from B. H. Hahn, University of Alabama, Birmingham, AL, and M. A. Martin, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD, respectively, through the NIH AIDS Research and Reference Reagent Program. 48 hours after transfection, virus was harvested, filtered (0.22 μm), and frozen at –80°C until use. Mice were injected i.p. with 0.2 ml PBS containing either YU-2 or NL4-3 at a tissue culture-infecting dose 50 of $2x10^6$. Infection was performed in a BL3 laboratory in accordance with IRB and the Institute of Veterinary Bacteriology (University of Bern) animal facility guidelines.

Analysis: Plasma HIV RNA concentrations were determined by Cobas Amplicor RT-PCR assay (Roche Diagnostics). p24 antigen levels in cell-culture supernatants were quantified by ELISA as described [191]. Human cell engraftment in mice was measured by flow cytometry as described [49]. Anti CXCR4 (12G5) and CCR5 (2D7) antibodies were from BD Pharmingen. Immunohistochemical stainings were performed in an automated Ventana Discovery Module (Ventana, Strasbourg, France). Paraffine sections were incubated in a 1:5 dilution of mouse monoclonal antibody to HIV-1 p24 (clone Kal-1; Dako). Staining for CD3 (SP7, Labvision) was performed according to Ventana protocols. Immunofluorescent stainings and microscopy were done as described [192]. Briefly, slides were sequentially incubated with: 1) mouse anti-HIV-1 p24 diluted 1:10 (DAKO Diagnostics AG, Zug, Switzerland), 2) 5 μg/ml of Cy3-conjugated goat anti-mouse immunoglobulin (Ig) (Amersham, Little Chalfont, GB); 3) 5

µg/ml of mouse IgG (Sigma, Buchs, Switzerland); 4) FITC-conjugated mouse anti-human CD68 (1:20; DAKO), 5) rabbit anti-FITC (1:2000; DAKO), and 6) 5 µg/ml Alexa 488-conjugated goat anti-rabbit Ig (Molecular Probes Europe BV, Leiden, The Netherlands) and 0.5 µmol/L of 4',6-diamidino-2-phenylindole (DAPI; Sigma). To assess non-specific binding, tissue from untransplanted and/or uninfected transplanted mice was stained as controls.

Immune responses: Human IgG levels in serum were measured on LC-Partigen plates (Behring, Marburg, Germany). HIV specific Western blot analysis was done using a commercially available kit (New Lav BlotI, BIO RAD). HIV-specific T cell responses were evaluated as previously described [193]: briefly, 10^6 splenocytes from HIV-infected and uninfected mice were pulsed with pools of overlapping peptides covering the HIV gag (NIH HIV-1 Consensus B Gag (15-mer) Peptides Set; pool I: Cat Nr. 7872 – 7933, pool II: Cat Nr. 7934 – 7994) or HIV nef (NIH HIV-1 Consensus B Nef (15-mer) Peptides Complete Set, Cat Nr. 5189) or with a 17-peptide pool (NIH Cat Nr. 7891, 7920, 7926, 7945, 7976, 7980, 7983 (gag), 5507, 5527, 5544, 5576 (pol), 6287, 6412, 6420 (env), 5172, 5183 (nef), 6078 (vpr)) at a concentration of $2x10^{-6}$M/peptide. One hour later, brefeldin was added for 5 hours. Splenocytes were stained with mAb against CD4, CD8, and intracellularly against IFN-γ using BD Cytofix/Cytoperm permeabilization/fixation kit (BD Pharmingen).

2.2.5 Acknowledgments

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2.3 Human T cell development and HIV infection in Human Hemato-Lymphoid System Mice

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Advances in generation of mice that upon human hematopoietic stem and progenitor cell transplantation develop and maintain human hemato-lymphoid cells fueled an already thriving field of research. We here focus on human T cell development and HIV infection in Rag2$^{-/-}\gamma_c^{-/-}$ mice transplanted as newborns with human CD34 positive cord blood hematopoietic stem and progenitor cells.

2.3.1 Introduction

For good reasons knowledge on human physiology and pathology is largely gained by observation, cautious, informed consent, and safety-oriented *in vivo* experimentation, and *in vitro* surrogate assays. Using these approaches, rigorous scientific proof is often impossible. Thus, progress in clinical research is mostly slow. Most societies agree on animal research using worms, flies, and small vertebrates given appropriate ethical consideration. Because of their high similarity with human beings, easy access and experimental feasibility, laboratory mice have become the main model for *in vivo* biomedical research reported in the vast majority of published work in high-ranking scientific journals. And for sure, the capturing slogan "They (laboratory animals) save more lives than 911 (the emergency call number)" is not an over-statement. However, although genome similarities are higher than naïvely expected, 65 million years of diverge in human and mouse evolution have shaped two species that differ substantially in size, lifespan, reproductive activity, and exposure to environmental challenges, as e.g., co-evolving species-specific infectious agents. Thus, also regarding hematology and immunology, mice are not men, accounting for one of the reasons that great achievements in mice are quite often lost in translational research [194,195].

Experimentation with human hemato-lymphoid system mice took off almost 20 years ago, paralleling increasing application of clinical hematopoietic stem cell transplantation for malignant disease and immunodeficiencies, as well as the rising HIV pandemic. Some basic requirements need to be met for hematopoietic cell engraftment, differentiation and function: cells need to find appropriate locations, must be supported by respective nurturing factors from the host environment, and must not be rejected. In cross-species transplantation, this requires immune-deficiency of the recipient, as well as cross-reactivity of homing molecules, and differentiation and survival factors, if not produced in sufficient amounts by transplanted cells themselve.

As the detailed history and state of the art in this field were recently reviewed [39-42]and major scientific contributions to the field are discussed in other chapters of this meeting book, we here briefly set the main focus on our work on T cell development and HIV infection using immunodeficient $Rag2^{-/-}\gamma_c^{-/-}$ mice (generated by M. Ito at the Central Institute for Experimental Animals, Japan), transplanted as newborns with human $CD34^+$ hematopoietic stem and progenitor cells.

2.3.2 Human T cell development and HIV infection in immunodeficient mice transplanted as newborns with human CD34 positive hematopoietic stem and progenitor cells.

One of the major improvements achieved by using newborn NOD/SCID$\gamma_c^{-/-}$ and Rag2$^{-/-}\gamma_c^{-/-}$ mice as recipients for human hematopoietic stem and progenitor cell grafts is efficient intra-thymic de novo development of human T cells [49,51,54,178]. In contrast to newborn transplantation, T cell development in adult NOD/SCID$\gamma_c^{-/-}$ recipients is less efficient but can be enhanced by exogenously adding human IL-7 [48,182]. Human T cells generated in the mouse thymus include in fairly physiologic ratios CD4$^+$ and CD8$^+$ T cells with a broad Vβ distribution, Foxp3$^+$ CD25$^+$ regulatory T cells, and γδ T cells. Mature T cells exit the thymus and home to secondary lymphoid organs. What is the MHC restriction and functionality of human T cells educated on a mouse thymic background? Data generated mostly in mice demonstrates that under normal developmental conditions positive selection preferentially occurs on cortical thymic epithelial cells, while both medullary epithelial cells and hematopoietic derived dendritic cells are involved in negative selection (e.g., reviewed in [196]). We did not observe human thymic epithelial cells in CD34$^+$ cell transplanted mice in line with expectations of epithelial cell germ-layer derivation, while dendritic cells of both mouse and human origin constitute the thymus. Thus, human developing thymocytes should in theory be positively selected on mouse epithelial cells, while negative selection might occur on both mouse and human MHC. If so, it would be reasonable to expect that T cells positively selected on mouse MHC would continue to preferentially interact in negative selection also with mouse MHC. However, under certain conditions hematopoietic offspring cells are likely involved in positive selection, as pointed out by several mouse to mouse transplantation studies [197,198]. Moreover, species-specific differences in thymic selection might exist, and human thymocyte-thymocyte MHC class II interaction, and thus at least some CD4 T cell selection, might occur on human MHC [199,200]. Upon T cell exit from the thymus, they depend on homeostatic factors, both MHC and cytokines for survival (e.g., reviewed in [201]). As in human hematopoietic stem and progenitor cell transplanted mice human MHC is only present on hematopoietic but no other tissues, presentation of both "self" and "non-self" peptides in the context of both MHC class I and II will depend on cellular tropism of these.

Given all considerations mentioned above, what experimental data on reactivity of mouse background generated human T cells has been reported? 1) human T cells isolated from mouse lymph nodes and spleen proliferated vigorously in mixed lymphocyte reactions

(MLRs) when stimulated with human allogeneic DCs, but weakly or not at all when stimulated with human autologous DCs. Proliferative response to mouse DCs was low, however, there was a small difference with stronger proliferation of T cells when stimulated with fully mismatched versus host mouse type dendritic cells [49]. 2) cytotoxic activity against human allogeneic target cells could be blocked using human MHC class I or II antibodies, respectively [51]. 3) some responses of human T cells in mice to *in vivo* infection of human B cells with Epstein-Barr virus are observed, however, in some cases human T cells were not capable to control EBV driven B cell proliferation [49]. 4) human T cells specific for viral epitopes were only observed in the context of mouse MHC upon infection of mice with influenza virus [40]; 5) when mature, mouse derived human T cells were transferred to non-transplanted $Rag2^{-/-}\gamma_c^{-/-}$ mice, we observed no relevant homeostatic cell expansion; similarly, high peripheral T cell turn-over rates and lack of long-term T cell maintenance was observed by other groups [40], suggesting that peripheral homeostatic T cell maintenance might not function appropriately, an issue that still needs to be resolved by more experimentation.

In summary, thus far no firm conclusion on the biology of T cell selection in this setting where the T cells are of human and the thymic stroma is of mouse origin can be drawn. It can only be suggested that T cell tolerance, i.e., possibly negative selection, for both autologous human and mouse MHC is achieved. Beyond, insufficient T cell responses due to no or weakly cross-species reactive co-stimulation and cytokine responses might account for additional difficulties in any human T cell response observed in this setting. In terms of appropriate T cell selection, the obvious solution is to replace mouse by human MHC components, creating at least for thymic selection, a similar situation as in mice co-transplanted with same donor fetal thymic tissue [57,202]. It will be of high interest to see if human T cell development will pursue in the absence of any tissue MHC, a model situation of allogeneic hematopoietic cell transplantation in human MHC deficiencies [57].

As in our human cord blood $CD34^+$ cell transplanted mice human T cells developed and seeded secondary lymphoid organs, we and concomitantly others, tested these mice, and mice co-transplanted with human thymus, as models for HIV infection [82,203-206]. We infected human hemato-lymphoid system mice i.p. with either CCR5 tropic or CXCR4 tropic HIV-1 strains. Irrespective of co-receptor selectivity, HIV RNA plasma copies peaked at 2-4 weeks after infection, comparable to HIV infection in man. Thereafter, viremia mostly stabilized at lower levels and was maintained for up to 190 days, the longest time followed. HIV generated in mice was functional, since supernatants of infected mouse-derived cell cultures propagated infection in primary human leucocytes. As in man, developing human $CD4^+$ thymocytes in

mice are mostly CXCR4-positive, but lack CCR5 expression, while peripheral $CD4^+$ T-cells express CXCR4 and/or CCR5. Thymic HIV infection was detected upon CXCR4 tropic infection, while secondary lymphoid organ infection occurred in both CXCR4- and CCR5-tropic virus infected animals (Fig. 1).

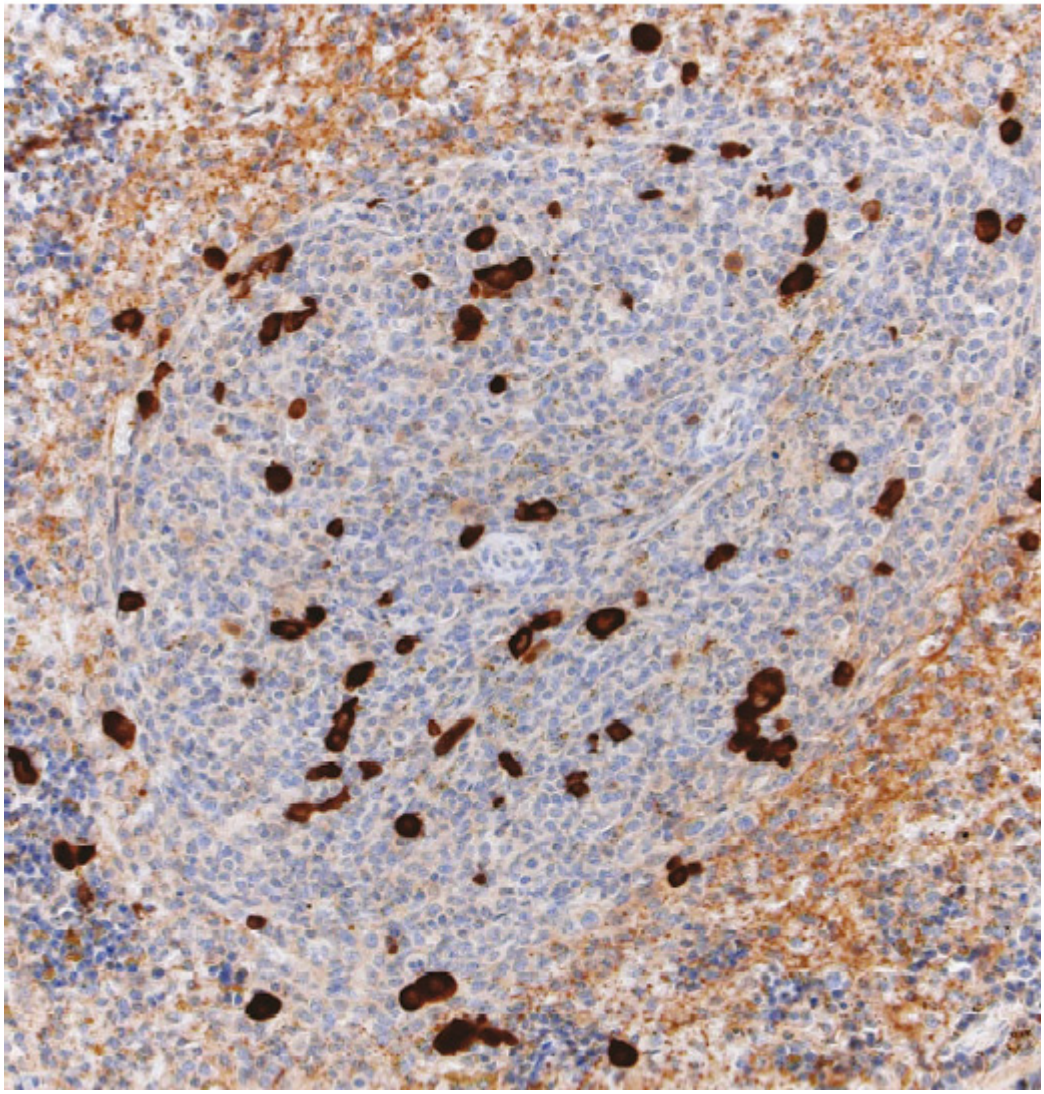

Fig. 1 $Rag2^{-/-}\gamma_c^{-/-}$ mice, transplanted as newborns with human $CD34^+$ cord blood cells and subsequently infected with HIV, develop lymphoid organ-disseminated, productive HIV infection. Histology shows HIV p24 staining preferentially localized in the white pulp area in the spleen of an HIV-infected mouse (18 days after infection with CCR5-tropic YU-2; Magnification 20x)

In both CXCR4- and CCR5-tropic strain infected animals productively HIV-infected cells, i.e., HIV-$p24^+$ cells, were mostly $CD3^+$ and only occasionally non T cells such as $CD68^+$ macrophages. In some mice with high numbers of productively infected cells, syncytium

formation occurred in both spleen and lymph nodes, a process observed in brains and lymphoid tissue of HIV infected individuals, likely associated with high viral replication, spreading, and CD4 cell loss. Overall, CXCR4-tropic HIV infection lead to more rapid blood $CD4^+$ cell loss than CCR5-tropic infection, reminiscent of CXCR4-tropic emergence of HIV strains in late-stage human HIV disease. One out of 25 infected mice mounted a HIV-specific IgG response detectable by standard clinical assays [82]. Furthermore, although based on limited data, we did not detect robust HIV-specific T cell responses, as determined by IFN-γ detection upon *in vitro* re-stimulation, in line with other concomitant reports [204]. Thus, although specific immune responses are observed, they thus far lack robustness, i.e., are not predictable in frequency and levels, prohibiting e.g., at this stage efficient pre-clinical vaccine testing. However, as is, this model will be valuable to study virus-induced pathology and to evaluate new non-adaptive immunity dependant approaches aiming to fight HIV.

2.3.3 Conclusions

It can be anticipated that basic and preclinical *in vivo* human immunology and infectious disease research will greatly benefit from improved, easy-to generate and broadly available human hemato-lymphoid system mice over the coming years.

2.3.4 Acknowledgements

We would like to thank the staff of the department of Obstetrics, Ospedale San Giovanni for cord blood supply, M. Ito for provision of BALB/c $Rag2^{-/-}\gamma_c^{-/-}$ mice, and the Swiss National Science Foundation and the Bill and Melinda Gates Foundation for research support.

2.4 Work in progress

2.4.1 RAG-hu mice as a screen for antiretroviral compounds that block sexual transmission.

Mucosal HIV transmission, i.e., vaginal and rectal transmission, is the primary mode of transmission worldwide, and understanding its biology is pivotal for ending the HIV pandemic. HIV research, in general, has long been hampered by the lack of an effective small animal model that replicates HIV infection in man. As a further "proof of concept," we propose to use RAG-hu mice to study mechanism(s) of vaginal HIV transmission, a particularly critical route of HIV infection, and to efficiently test compounds / vaccines for blocking mucosal HIV transmission in a preclinical model.

Progesterone treatment thins the mulitlayer, stratified squamous epithelium.

Vaginal transmission studies in monkey models with SIV have frequently pretreated the challenged animals with progesterone, with the purpose of altering the cellular architecture of the vaginal epithelium to facilitate viral transmission. In addition, this treatment synchronizes the oestrous cycle among experimental animals. Experiments with hu-PBL-SCID mice revealed that vaginal HIV transmission is only successful with prior administration of progestin [207,208]. Therefore, we investigated the influence of progesterone treatment on the vaginal epithelium of $Rag2^{-/-}\gamma_c^{-/-}$ mice as related to their histological appearance (Fig 1).

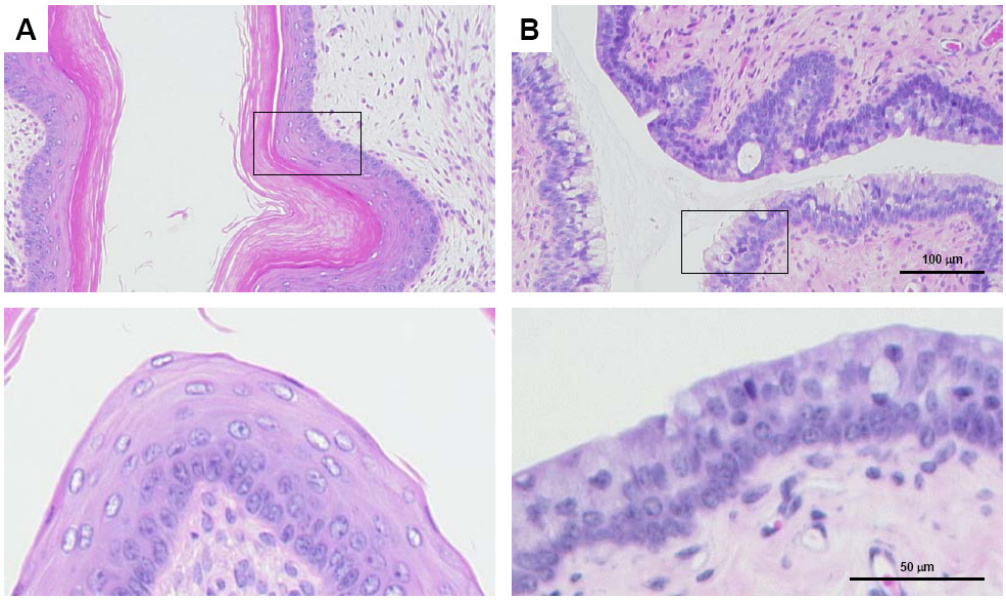

Fig. 1: Progesterone treatment of $Rag2^{-/-}\gamma_c^{-/-}$ results in a thinning of the multilayer, stratified, squamous epithelium (B) as compared to the vagina of untreated mice (A) Mice were treated subcutaneously with 2.5 mg progestin (Depo-Provera, Pfizer).

RAG-hu mice have human cells in the epidermal layer of the vagina.

In macaques HIV is first detected in specialized cells of the lymphoid system, namely dendritic cells, in the vagina soon after inoculation with SIV. The conventionally used mouse models (i.e., hu-PBL-SCID, SCID-hu) lack human target cells in the vagina that free virus could infect. In contrast, it might be highly likely that RAG-hu mice have human cells in the epidermal layer of the vagina. We therefore tested whether RAG-hu mice have human cells in the epidermal layer of the vagina (Fig 2). Indeed, we detected the presence of some human cells by immunohistochemical staining.

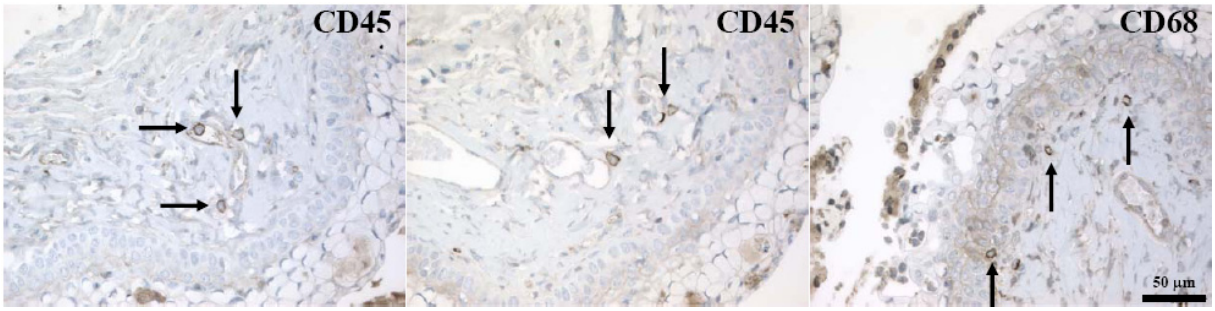

Fig. 2: RAG-hu mice have human cells in the epidermal layer of the vagina (arrows). Vaginal tissue of RAG-hu mice was stained for the panhuman leukocyte marker CD45 or the macrophage marker CD68.

Cell-associated viral transmission by vagina irrespective of co-receptor usage.

Vaginal transmission of cell-associated virus is more efficient than that of cell-free virus. Moreover, it has been reported that HIV strains using the chemokine receptor CCR5 is much more efficiently transmitted than HIV strains using the chemokine receptor CXCR4 for cellular entry.

We, thus, tested in a first step vaginal HIV transmission with cell-associated HIV. RAG-hu mice allow for cell-associated HIV transmission by vagina irrespective of co-receptor usage (Fig 3). We are currently investigating the transmission of cell-free HIV.

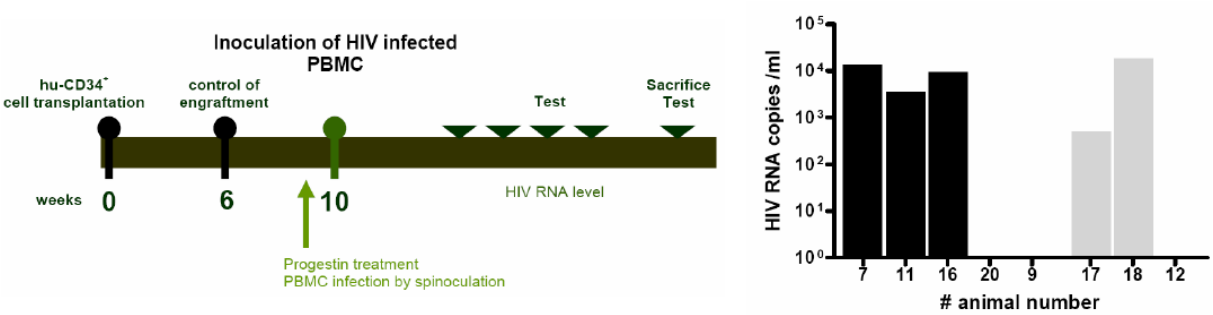

Fig. 3: RAG-hu mice allow for cell-associated viral transmission by vagina irrespective of co-receptor usage. Seven days after progestin treatment, mice were anaesthetized and $2x10^6$ HIV infected human peripheral leukocytes (huPBL) per mouse were inoculated by the vaginal route. Three weeks later, HIV RNA load in peripheral blood was determined by Cobas Amplicor assay. Five mice were inoculated vaginally with human PBL infected with a CCR5-tropic strain (Yu-2; black bars) and three mice were inoculated with human PBL infected with a CXCR4-tropic strain (NL4-3; gray bars).

Perspective

A mouse model of vaginal transmission of cell-associated and cell-free virus would provide a simple and inexpensive tool to identify antiretroviral compounds that block sexual transmission. Blocking heterosexual transmission of HIV would be a milestone in the fight against the HIV pandemic. The long-term goal would be to make this model available to the research community for wider testing of various compounds for inhibiting HIV vaginal transmission.

We would like to emphasize that this humanized mouse model is susceptible to the vaginal transmission of HIV strains using either CCR5 or CXCR4. Consequently, our model may help to elucidate whether the preferential transmission of CCR5-tropic viruses in the clinical setting reflects preferential movement of CCR5-utilizing virus-infected cells across the mucosal barrier or an enhanced ability of these cells for the spread of HIV.

2.4.2 APOBEC3: its role as restriction factor in HIV infection.

Although antiretroviral therapy has dramatically ameliorated the situation of people living with HIV, the pandemic continues resulting in more than 40 million people infected, almost 5 million people newly infected and 3 million deaths in 2005. Thus, new therapeutic strategies are urgently needed [1].

A powerful mechanism of innate immunity has recently been discovered, in which APOBEC proteins pose powerful barriers to the replication of HIV and other retroviruses [209,210]. APOBEC are a family of cytidine deaminases. They hinder HIV replication mainly by hyperdeamination of the nascent HIV DNA strand in newly infected cells [211,212]. HIV on its part has evolved Vif (viral infectivity factor), which mediates APOBEC degradation [213]. The balance between APOBECs and Vif is highly likely to be a crucial determinant for the outcome of HIV infection; targeting APOBEC/Vif interaction might represent a promising therapeutic approach [214], which in turn necessitates a detailed understanding of its molecular mechanisms. Little is known, however, about the signalling pathways involved in induction of APOBEC expression. Moreover, APOBECs have been examined exclusively *in vitro* by using predominantly cell lines; the importance of APOBECs in an *in vivo* system has yet to be explored.

Working Hypothesis: We hypothesize, that APOBEC3 proteins are key players in the innate immune response against HIV and are anti-viral active throughout the course of HIV disease.

The main goal of this study is i) to investigate APOBEC3's anti-viral effects against various HIV strains *in vitro* and in particular *in vivo*, and ii) to what extent APOBEC3's anti-viral activity may be enhanced by cytokines and ligands triggering pattern recognition receptors, in particular ligands triggering Toll-like receptors. RAG-hu mice have significant advantages over existing xeno-chimeric models and represent an excellent tool to approach APOBEC biology in an *in vivo* setting.

3

Impact of TLR7 triggering on immune activation and dysfunction

This part is submitted as Baenziger-Heikenwalder et al. 2008

3. Triggering TLR7 in Mice Results in Immune Activation and Disruption of the Lymphoid System, Resembling HIV-Associated Pathology

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Chronic immune activation is a major cause for progressive immunodeficiency in infection by human immunodeficiency virus-1 (HIV). The underlying trigger, however, remains largely unknown. HIV single-stranded RNA serves as a potent immune activator by triggering Toll-like receptor (TLR) 7/8. We hypothesized that sustained TLR activation induces immune activation and thereby may contribute to progressive immunodeficiency. The synthetic compound R848 was used to explore the effects of sustained TLR7 triggering on the murine lymphoid system. Sustained TLR7 activation induced an immunopathology reminiscent of progressive lymphoid destruction in HIV disease. We show profound lymphopenia, elevated proinflammatory cytokines, splenomegaly, contracted lymphoid subsets, and altered lymphoid microarchitecture with enlarged B- and T-cell zones and reduced marginal zone B lymphocytes. Upon exposure to inactivated vesiculo-stomatitis virus, antibody production was abolished, although splenic lymphocytes were activated and total IgG was elevated. These TLR7-specific effects were partly mediated by interferon regulator factor 7 and type I interferon receptor signalling. Our data imply that HIV itself may directly contribute to immune activation and dysfunction by stimulating TLR7. Notably, HIV virions and single-stranded RNA reach high copy numbers in plasma, although only a minor fraction of these particles is infectious. Manipulation of TLR7 signalling may thus be a potential antiviral strategy.

3.1 Introduction

Progressive $CD4^+$ T-cell depletion and chronic immune activation are hallmarks of human immunodeficiency virus-1 (HIV) infection. Chronic immune activation includes aberrant cytokine production, redistribution of lymphocyte subpopulations, polyclonal B-cell activation, increased T-cell turnover, and increased numbers of activated T cells.

Several observations suggest a crucial role of chronic immune activation in HIV pathogenesis. i) Increased numbers of activated $CD4^+$ T cells expressing the activation markers CD69, CD25, and MHC class II as well as activated $CD8^+$ T cells expressing the activation marker CD38 correlate with HIV disease progression and $CD4^+$ T-cell loss in untreated infection [90-92]. Notably, CD38 expression on $CD8^+$ T cells is considered to be a better prognostic marker than $CD4^+$ T-cell counts or peripheral HIV RNA load [104]. ii) Immune activation is absent in non-pathogenic simian immunodeficiency virus (SIV) infection. In particular, the natural hosts of SIV, sooty mangabeys and African green monkeys, show only minimal increases in immune activation and rarely progress to immunodeficiency despite high levels of viral replication [16,215]. iii) Immune activation facilitates HIV infection of T cells by promoting co-receptor, adhesion molecule, and nuclear factor (NF)-κB expression [96-98]. iv) Persistent immune activation in mice that constitutively express CD70, can induce lethal T-cell immunodeficiency [216].

The precise mechanism and underlying trigger by which HIV causes immune activation remain poorly understood. Immune activation in pathogenic SIV and HIV infections might be partly explained by evolutionary changes in Nef function. Schindler and colleagues [108] noted that the Nef from nonpathogenic SIV strains mediates downmodulation of the host T-cell receptor and thereby suppressed T-cell activation, whereas Nef from HIV did not. However, this hypothesis remains controversial [109]. Alternatively, T-cell recognition of HIV antigens, gp120- and Nef-mediated signalling [217,218], and regulatory $CD4^+$ T-cell depletion [219] have also been proposed as major contributors to immune activation. Regulatory T cells are important for suppression of T-cell activation.

Toll-like receptor (TLR) activation may not only enhance antiviral immunity by activating the immune system (reviewed in [220]), but also play a pivotal role in aberrant immune activation in HIV infection [110]. TLRs belong to the family of pattern recognition receptors, are essential in innate immunity in mammals by recognizing conserved patterns of microorganisms (PAMP), are selectively expressed on cells of the immune system [111], and are classified according to the types of PAMPs they recognize (reviewed in [112]). TLR3, 7, 8 and 9, are localized

intracellularly and detect nucleic acids derived from viruses or bacteria. In particular, TLR7/8 sense viral single-stranded (ss) RNA [221].

Brenchley et al. proposed circulating microbial products (TLR4 ligands), likely derived from the gastrointestinal tract upon breakdown of the mucosal barrier, as a cause of HIV-related systemic immune activation [113]. Immune activation correlates with HIV viremia and declines rapidly after initiation of HAART and long before peripheral $CD4^+$ T-cell recovery [114,115]. These observations suggest a direct role for viral replication products in immune activation [116,117]. In this context, HIV ssRNA is likely to contribute directly to immune activation: human TLR7/8 and murine TLR7 recognize HIV ssRNA and synthetic imidazoquinoline-like molecules [114,118,119]. Importantly, HIV ssRNA has been shown to encode for multiple TLR7/8 ligands that indeed induce strong immune activation *in vitro* [114].

In natural HIV infection, the immune system fails to eliminate the virus, resulting in persistent ssRNA and virion production, although only a minor fraction of these virions (i.e., 1/60,000) is infectious [222]. Nonetheless, these non-functional viral particles may have a biological significance because they constantly induce TLR signalling. We recently showed that constant triggering of TLRs is harmful to the immune system. Repetitive triggering of TLR9 dramatically altered the morphology and functionality of mouse lymphoid organs [223].

Here we hypothesized that sustained immune activation through TLR7/8 is a main driver for chronic immune activation and thereby contributes to the progressive immunodeficiency observed in HIV infection. To explore the effects of sustained TLR7 triggering on the molecular and cellular components of the lymphoid system of mice, we used the synthetic compound R848, which triggers TLR7/8 in a manner similar to HIV ssRNA [114,118,120]. Unlike in humans, TLR8 is nonfunctional in mice [224]. We demonstrate that chronic triggering of TLR7 in mice results in immune activation, functional impairment and disruption of the lymphoid system reminiscent of HIV-associated pathology.

3.2 Results

Continuous stimulation of TLR7 induced sustained lymphopenia and increased neutrophil and monocyte numbers.

To assess the effects of sustained TLR7 triggering *in vivo,* C57BL/6 mice were treated with the R848 daily for up to 42 days. We hereby aimed to replicate the persistent nature of HIV infection with sustained exposure of lymphoid tissue to HIV ssRNA burden.

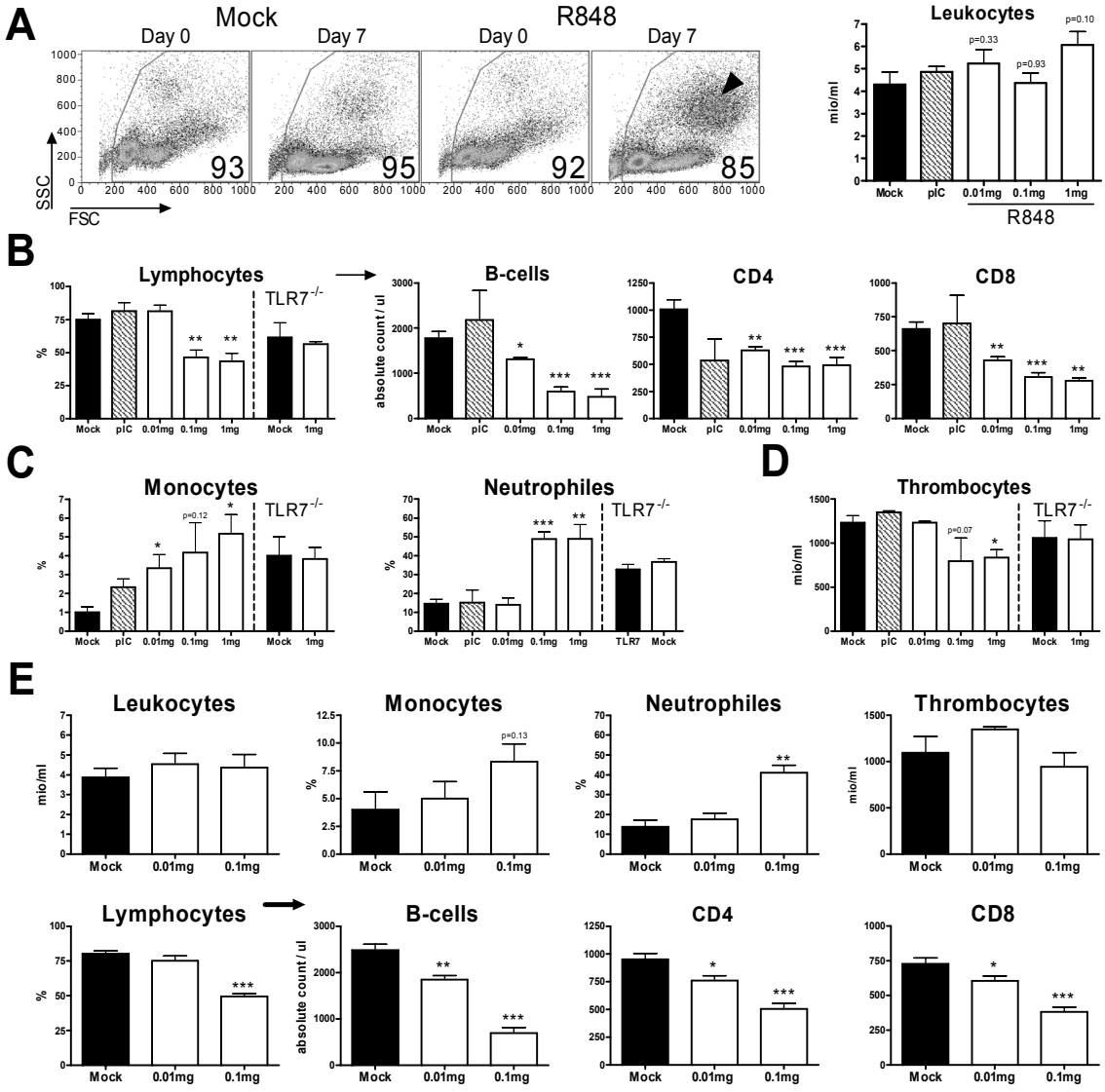


Fig. 1. Sustained stimulation of TLR7 dramatically induced sustained lymphopenia and increased neutrophil and monocyte numbers. (A–D) Mice were treated with the TLR7 ligand R848 in a daily fashion. After 7 days of treatment, blood was analyzed for the presence of leukocytes, lymphocytes, monocytes, neutrophils, and thrombocytes 1 h after the last injection. (A) R848 treatment did not affect absolute numbers of circulating leukocytes. Left: 1 mg R848/kg/day; analysis by flow cytometry before and after treatment. Numbers signify percentage of viable leukocytes as determined by FSC/SSC characteristics. Arrowhead indicates monocytes and neutrophils (i.e., large granular cells with high FSC/SSC properties). Right: analysis by hemocounter. (B) Total lymphocytes and all major lymphocytic cellular subsets (i.e., $B220^+$ B cells, $CD4^+$, and $CD8^+$ T cells) were massively decreased, whereas (C) monocyte and neutrophil numbers increased. Effects are dose- and TLR7-dependent. Lymphocyte, monocytes and neutrophils numbers were determined mechanically; absolute numbers of lymphocyte subpopulations were determined using BD truCOUNT technology. (D) Thrombocyte numbers were determined with hemocounter. (E) Similar analysis after 21 days of TLR7 stimulation. Error bars indicate standard error. Data of at least three individually measured animals are shown. pIC = polyI:C.

Mice treated with R848 showed no loss of body weight or signs of overt toxicity: liver enzymes and markers of renal, hepatic, cardiac, and pancreatic functions remained the same as those in PBS-treated control mice (data not shown). In general, no or only minor blood-parameter changes were observed at the lowest dose of 0.01 mg/kg/d R848, whereas pronounced effects were observed at 0.1 and 1 mg/kg/d R848. TLR7 triggering at neither dose did not alter absolute numbers of circulating leukocytes as determined by flow cytometry (Fig. 1A, left) and by an automatic hemocounter (Fig. 1A, right). Similar data were observed when mice were given a single dose and analyzed after 30, 90, and 180 min (data not shown), in contrast to recently reported data [225].

In contrast, total lymphocytes and all major lymphocytic cellular subsets (i.e., $B220^+$, $CD4^+$, and $CD8^+$ cells) were significantly decreased after 7 days of TLR7 triggering in a dose-dependent fashion (Fig. 1B), consistent with reported data [114,226]. In contrast, absolute and relative monocytes and neutrophils numbers dramatically increased (Fig. 1A, C and data not shown). Blood from R848-treated mice also showed a tendency towards reduced thrombocyte counts (Fig. 1D), which is frequently associated with HIV infection [227]. The detected changes in blood are TLR7 specific and not a universal phenomenon in response to triggering TLRs, since equivalent triggering of TLR3 by its ligand polyI:C did not lead to changes in any of the above described parameters in C57BL/6 mice when compared to untreated controls. Moreover, the observed effects were nearly completely abolished in $TLR7^{-/-}$ mice. In contrast, type I interferon (IFN)-mediated signalling via the IFN receptor (IFNAR) and interferon regulatory factor (IRF) 7 signalling seemed not to be critically involved, since white blood cell counts were similar in R848-treated $IFNAR^{-/-}$ or $IRF7^{-/-}$ mice, when compared to C57BL/6 mice (Fig. 2).

We next addressed whether chronic TLR7 triggering over an expanded time period with low doses would have the same effect as the 7-day high-dose regimen. Treatment with R848 for 21 and 42 days induced similar effects. No or only minor effects were detected at the lowest dose of 0.01 mg/kg/d, while profound lymphopenia and increased neutrophil and monocyte numbers were seen at the 10-fold dose (Fig. 1E and not shown). In conclusion, sustained triggering of TLR7 dramatically alters white blood cell counts, including profound lymphopenia and increased neutrophil and monocyte levels.

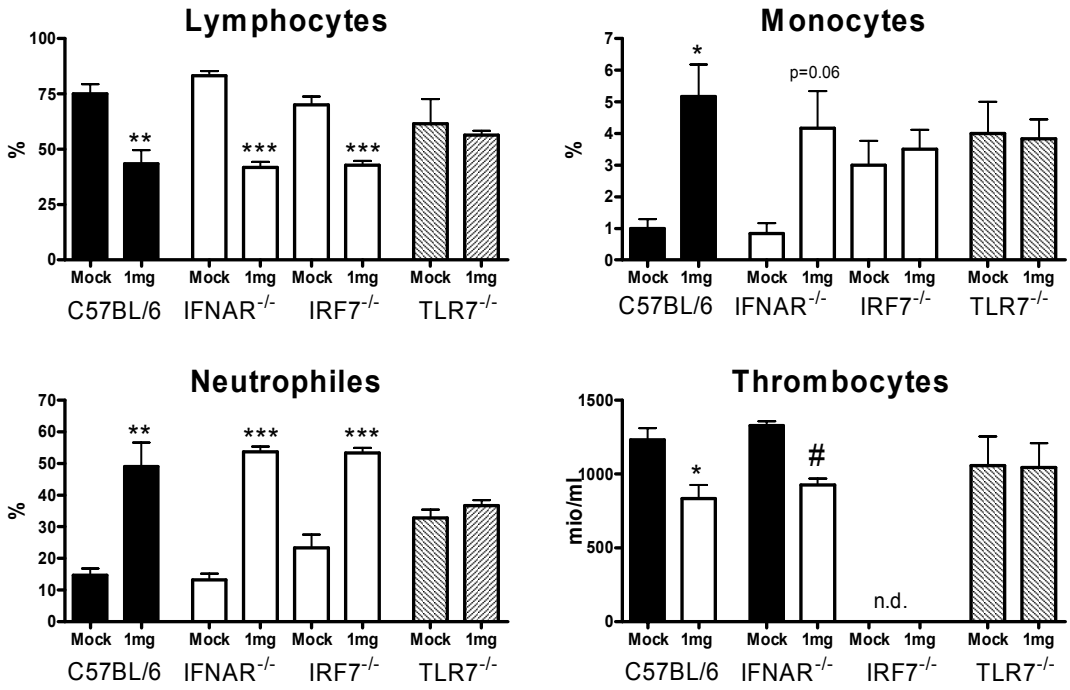


Fig. 2. R848-induced lymphopenia and increased neutrophil and monocyte numbers are TLR7 dependent. Mice were treated with 1 mg/kg/d R848 for 7 days. Blood lymphocytes, monocytes, neutrophils, and thrombocytes numbers were determined with a hemocounter one hour after the last injection. Effects are TLR7 dependent but IFNAR and IRF 7 signalling seems not to be critically involved because effects in R848-treated mice lacking IFNAR or IRF7 were similar as in wildtype mice. Data of at least 3 individually measured animals are shown but IFNAR$^{-/-}$ thrombocyte counts (#) where only 2 animals are shown.

Sustained triggering of TLR7 causes reduced antigen-specific humoral immune response and immune activation.

Defective humoral immunity is a hallmark of HIV infection [228,229]. Gunzer et al. suggested that the R848-induced lymphopenia and the concurrent transient decrease of immune cells might lead to a transient immunodeficiency with a corresponding increase in susceptibility to infections [225]. On the other hand, TLR agonists represent promising candidates to efficiently induce specific antibody responses.

We sought to determine the effect of chronic TLR7 activation on humoral immune responses. After 7 days of R848 treatment (1 mg/kg/d) mice were immunized intravenously with $1x10^6$ plaque forming units (PFU) of ultraviolet-inactivated vesicular stomatitis virus (UV-VSV). Antigen exposure is a major determinant for a subsequent VSV-specific humoral immune response. Since the milieu generated by sustained triggering of TLR7 may affect the strength

of VSV replication and thus the antigen exposure, we used inactivated VSV that guaranteed the same antigen-load in the mock- and R848-treated mice. All mice showed an IgM-IgG class switch between days 3 and 7 after immunization (Fig. 3A). However, triggering TLR7 caused reduced IgM titers at day 3 after UV-VSV challenge and a striking reduction in immunoglobulin class switch with very low IgG titers 7 and 10 days after UV-VSV challenge. Thereafter, IgG titers slowly recovered, perhaps because stopping R848 treatment upon immunization led to gradual reversion of TLR7-mediated effects and because antigen was gradually released from its local depot. Nonetheless, VSV-specific IgG titers were still slightly reduced 20 days after immunization.

B-cell activation, with the consequent hypergammaglobulinaemia, is one of the most characteristic features of the intricate immune impairment observed in HIV infection [228]. This phenomenon involves all immunoglobulin subclasses and results in the augmented production of HIV-specific and, mostly, HIV-unrelated antibodies [230]. By contrast, chronic hyper-activation of B lymphocytes could lead to B-cell exhaustion or anergy and to concomitant low levels of immunoglobulin. We tested whether sustained triggering of TLR7 negatively affected total IgG levels, which in turn could explain the attenuation of VSV-specific IgG levels. UV-VSV inoculation and subsequent antibody response resulted in a twofold increase of total IgG on day 20 after immunization in both mock- and R848-treated mice (Fig. 3B). Strikingly, total IgG levels were increased by approximately 50% in R848-treated mice as compared to mock-treated mice after 7 days of R848 treatment. Thus, immunosuppression was not due to a general incapability of B cells to produce IgG.

Splenic lymphoid subpopulations from R848-treated C57BL/6 mice displayed an activated phenotype as determined by an increased mean fluorescence intensity of the early activation marker CD69 and an increased percentage of CD69-expressing cells (Fig. 3C and D). The extent of activation was dose-dependent (Fig. 3C). In particular, more than threefold increase in the percentage of $CD69^+$ $B220^+$ B lymphocytes and $CD69^+$ $CD8^+$ T cells was detected in C57BL/6 mice treated with 1 mg/kg/d R848 for 7 days. $CD69^+$ $CD4^+$ T cells were increased twofold (Fig. 3D). CD69 upregulation was not observed in $TLR7^{-/-}$ mice and less pronounced in $IFNAR^{-/-}$ or $IRF7^{-/-}$ mice. Analysis of mesenteric lymph nodes revealed similar results (not shown). Surprisingly, lymphoid subsets from the blood showed no signs of CD69 upregulation (not shown), indicating a particular activation pattern that occurred locally in secondary lymphoid organs. Moreover, splenic T cells did not upregulate activation marker CD44 or downregulate cell-adhesion molecule CD62L.

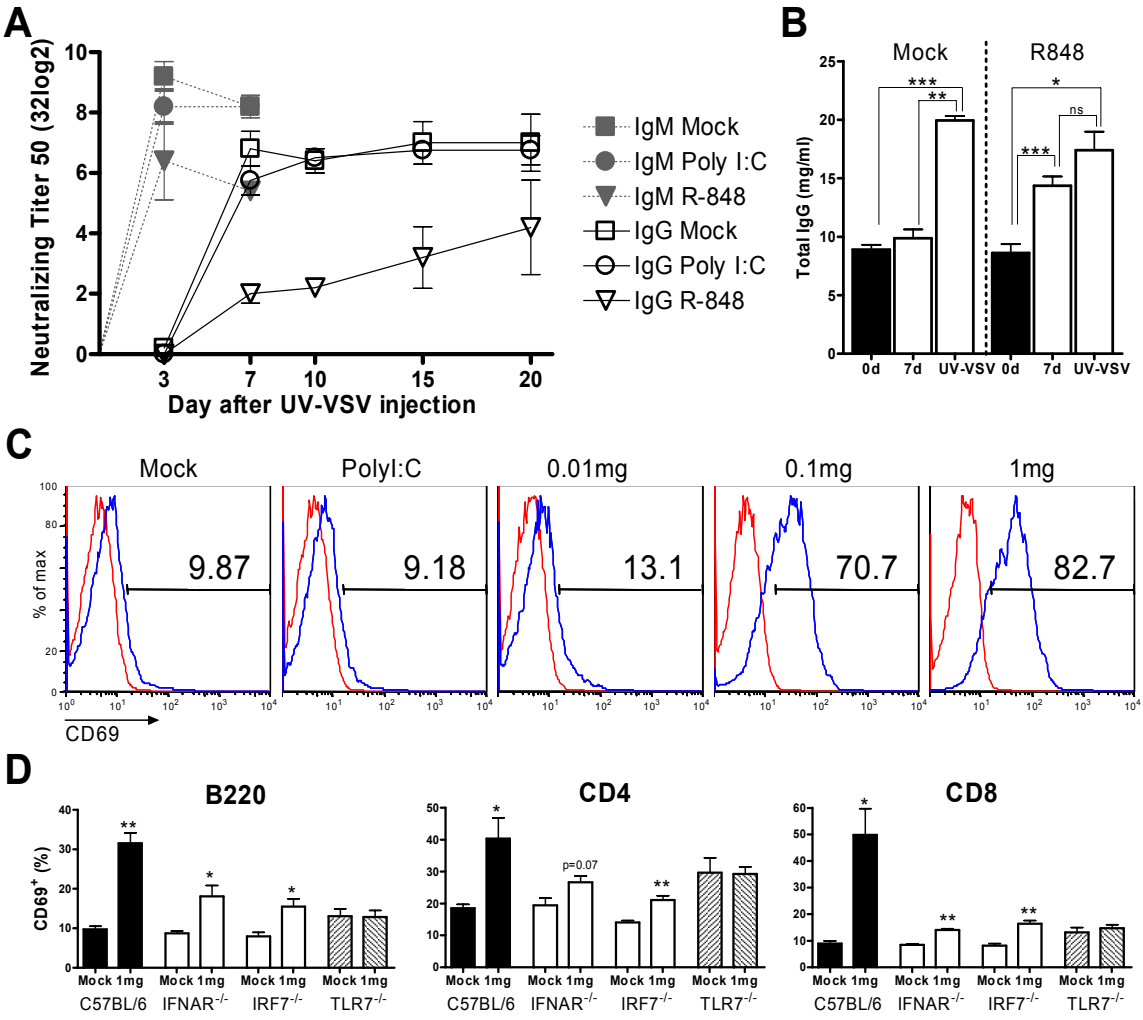


Fig. 3. Chronic TLR7 triggering results in a weakened antigen-specific humoral immune response despite immune activation. (A) Attenuation of humoral immune response after treatment with UV-VSV. Mice were treated with PBS or 1 mg/kg/day R848 for 7 days and subsequently immunized with UV-VSV. Neutralizing IgM and IgG titers were monitored for the following 20 days. N = 5 (B) Immunosuppression is not due to a general incapability of B cells to produce IgG. Total IgG levels were measured at day 0, after treatment with 1 mg/kg/day R848 for 7 and 20 days after UV-VSV inoculation. Means were compared using paired t test. ns = not significant. N ≥ 5 (C–D) Splenic lymphoid subpopulations from R848-treated animals displayed an activated phenotype after 7 days of treatment. Receptor expression was analyzed by flow cytometry. (C) Dose-dependent increase of CD69 mean fluorescence intensity on $B220^+$ B cells. Red and blue lines indicate cells stained with isotype or specific mAb, respectively. One representative mouse from each group is shown. (D) TLR7-dependent increase in the percentage of lymphoid subsets expressing CD69. Error bars indicate standard error. N ≥ 5.

Effects were not cumulative, because the degree of CD69 upregulation was similar after 7, 21, or 42 days. When mice were treated with 0.1 mg/kg/d R848 for 21 and 42 days, we observed a consistent two- to threefold increase of splenic CD69 expression. Long-term treatment with 0.01 mg/kg/d R848 for 42 days had only minor effects (not shown).

In conclusion, our data suggest that sustained triggering of TLR7 induces a particular lymphocyte activation in secondary lymphopid organs, but prevents an adequate antibody response against UV-VSV.

Sustained TLR7 triggering disrupts the lymphoid structure.

We further elucidated the reasons for the observed TLR7-stimulation dependent immunosuppression. Since efficient immune responses rely on intact lymphoid organs, we investigated splenic follicular and germinal centre microarchitecture upon sustained TLR7 stimulation. Treatment with 0.1 or 1 mg/kg/d R848 led to malformed and severely enlarged white-pulp follicles (Fig. 4). Moreover, sustained TLR7 triggering resulted in a dose-dependent hypertrophy of B-cell follicles as well as $CD8^+$ and $CD4^+$ T-cell zones in the splenic white pulp (Fig. 4A). Conversely, marginal zone B lymphocytes were strongly reduced (B220 and CD21/35; arrowhead). Macrophages, in particular marginal zone metallophilic MOMA-1^+, which appear to play a role in the initial response to systemic infection [231], and $F4/80^+$ red pulp macrophages, which are extensively involved in the clearance of senescent erythrocytes [231], did not seem to be affected (Fig. 4B). Repetitive treatment with PBS, polyI:C or 0.01 mg/kg/d R848 did not induce any derangement of lymphoid micro-architecture (Fig. 4).

Again, the effects detected were likely to be persistent rather than cumulative or mitigated. When mice were treated for a longer period, splenic microarchitecture was not further compromised. After 21 and 42 days of 0.01 mg/kg/d R848 treatment, spleen microarchitecture was only slightly disturbed, and B- and T-cell areas were nearly unaltered, in contrast to a massive enlargement in 0.1 mg/kg R848-treated mice (Figs. 5A/B and 6A/B) which was already seen after 7 days.

Disruption of the lymphoid structure was TLR7 dependent since $TLR7^{-/-}$ mice showed neither disrupted white pulp follicles with enlarged B- and T-cell zones nor diminished marginal zone B lymphocytes after 7 days of treatment with 1 mg/kg/d R848 (Fig. 7A/B). $IRF7^{-/-}$ mice displayed no obvious symptoms of lymphoid follicle malformation. In contrast, $IFNAR^{-/-}$ mice showed some degree of inflated follicles with loosened rings of marginal zone B cells (Fig. 7A/B), indicating that IFNAR and IRF7 signalling partly contributed to R848-mediated pathology.

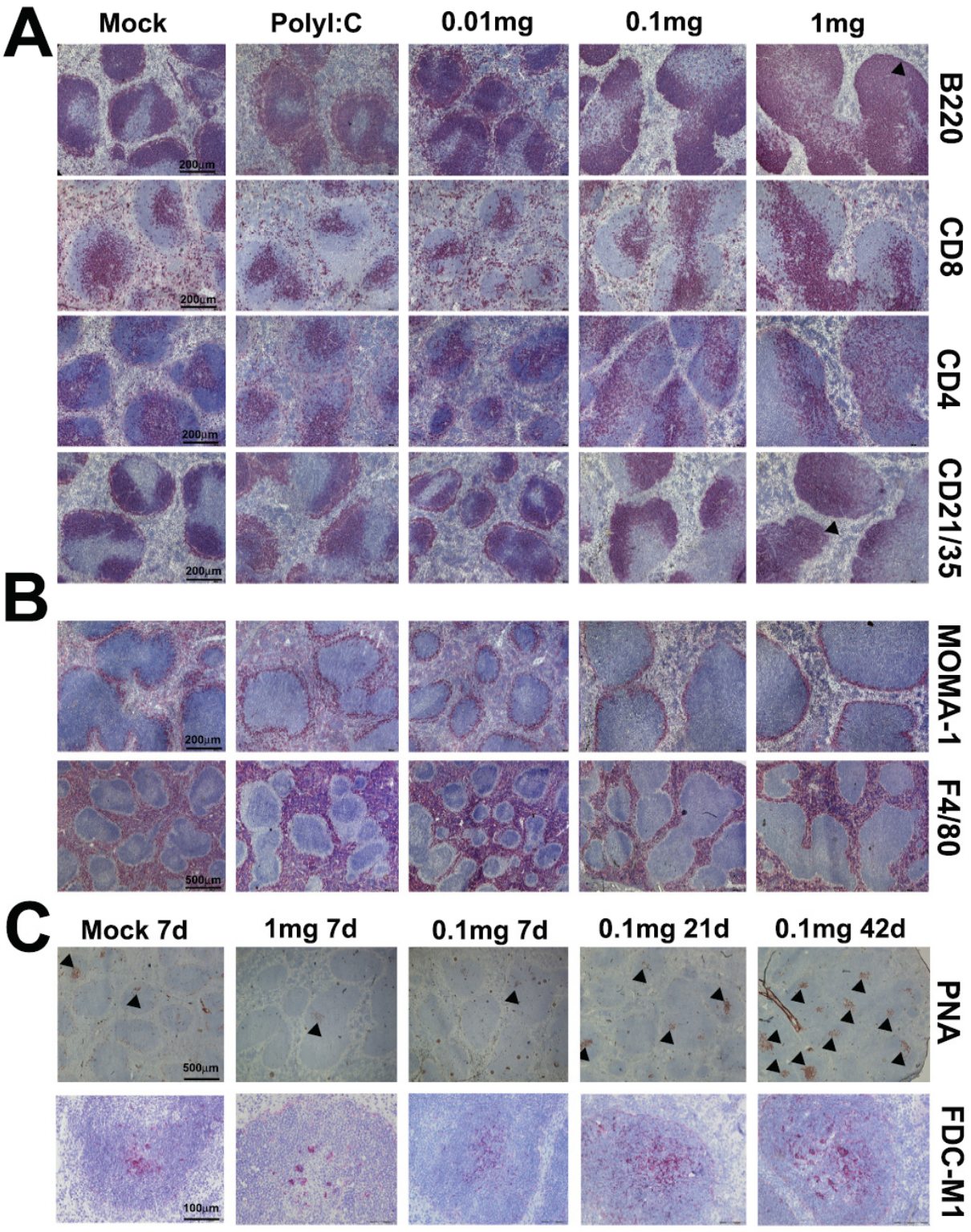


Fig. 4. Disruption of the lymphoid structure after 7 days of chronic TLR7 stimulation. Spleen cryosections of C57BL/6 mice treated for 7 days with R848. (A) Applied dose correlated with malformation of splenic lymphoid follicles with enlarged T- (CD4 and CD8) and B- (B220) cell zones, and reduced marginal zone B lymphocytes (B220 and CD21/35; arrowhead). (B) MOMA-1^+ and F4/80^+ macrophages were not affected. (C) PNA^+ germinal centres (arrowheads) and FDC-$M1^+$ networks were reduced at day 7 but not at day 21 or 42 of treatment. R848 concentrations (mg/kg/day) as indicated. $N \geq 3$.

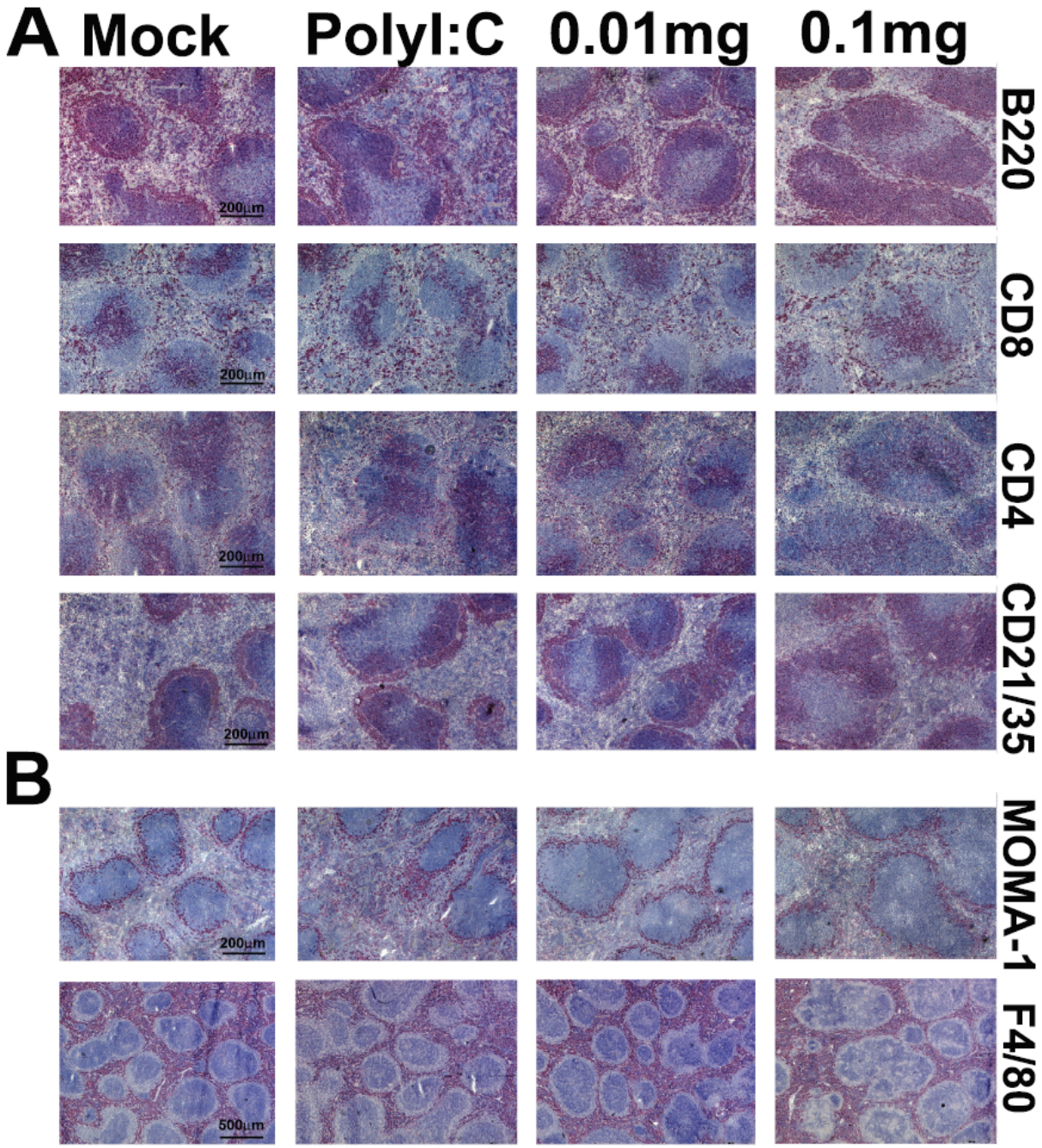


Fig. 5. Disruption of the lymphoid structure is not further compromised after 21 days of chronic TLR7 stimulation. (A) Spleen cryosections of C57BL/6 mice showing a dose dependent malformation of lymphoid follicles with enlarged T and B cell zones, and reduced marginal zone B-lymphocytes. Lymphoid disruption was not further compromised after 21 days of treatment as compared to the 7 day-treatment (see also Figure 4). (B) MOMA-1^{+} and F4/80^{+} macrophages were not affected. (C). N ≥ 3.

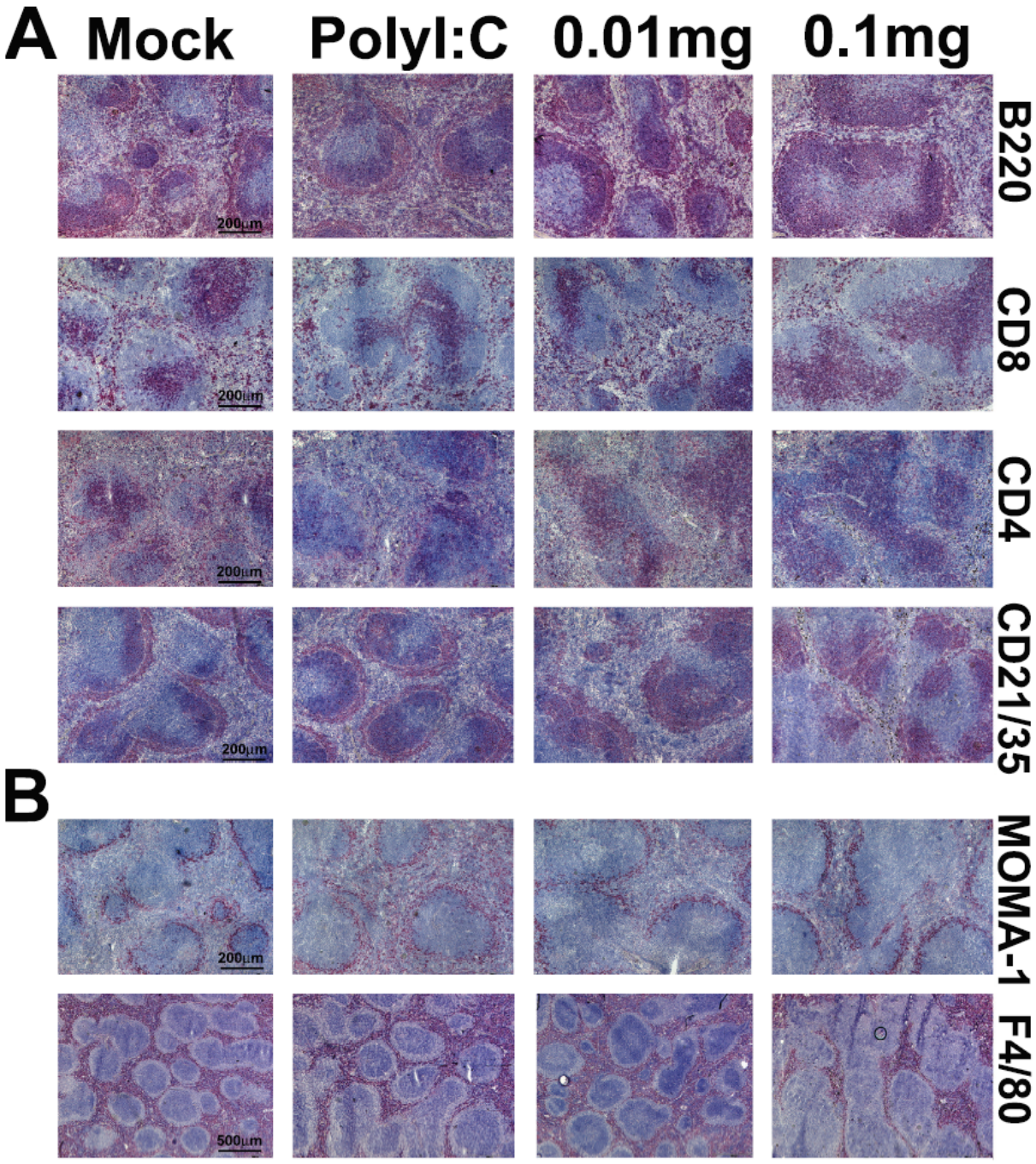
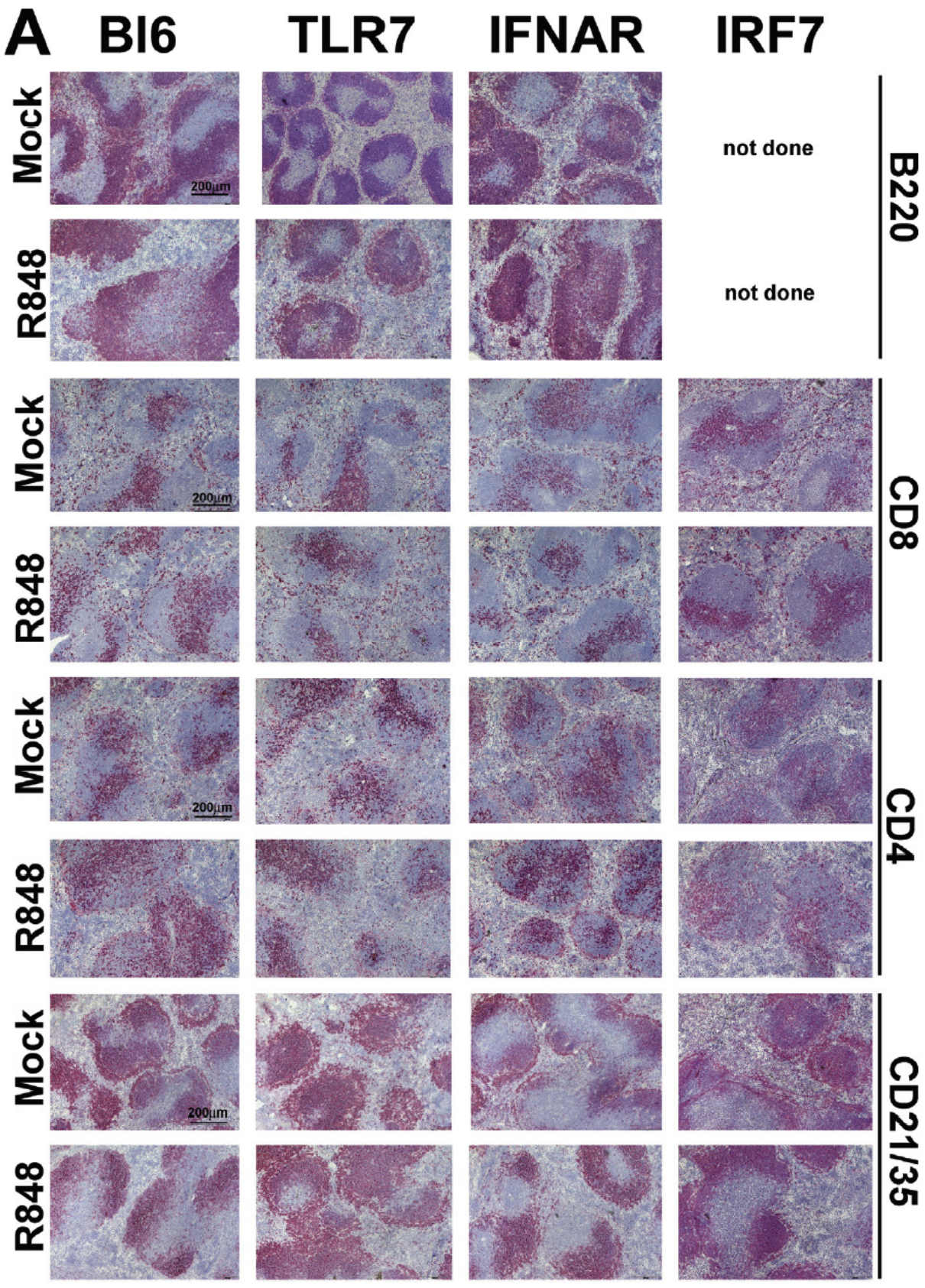


Fig. 6. Disruption of the Lymphoid Structure is not further compromised even after 42 days of chronic TLR7 stimulation. (A-B) See legend figure 5 for details.


A
BI6
TLR7
IFNAR
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Mock
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not done
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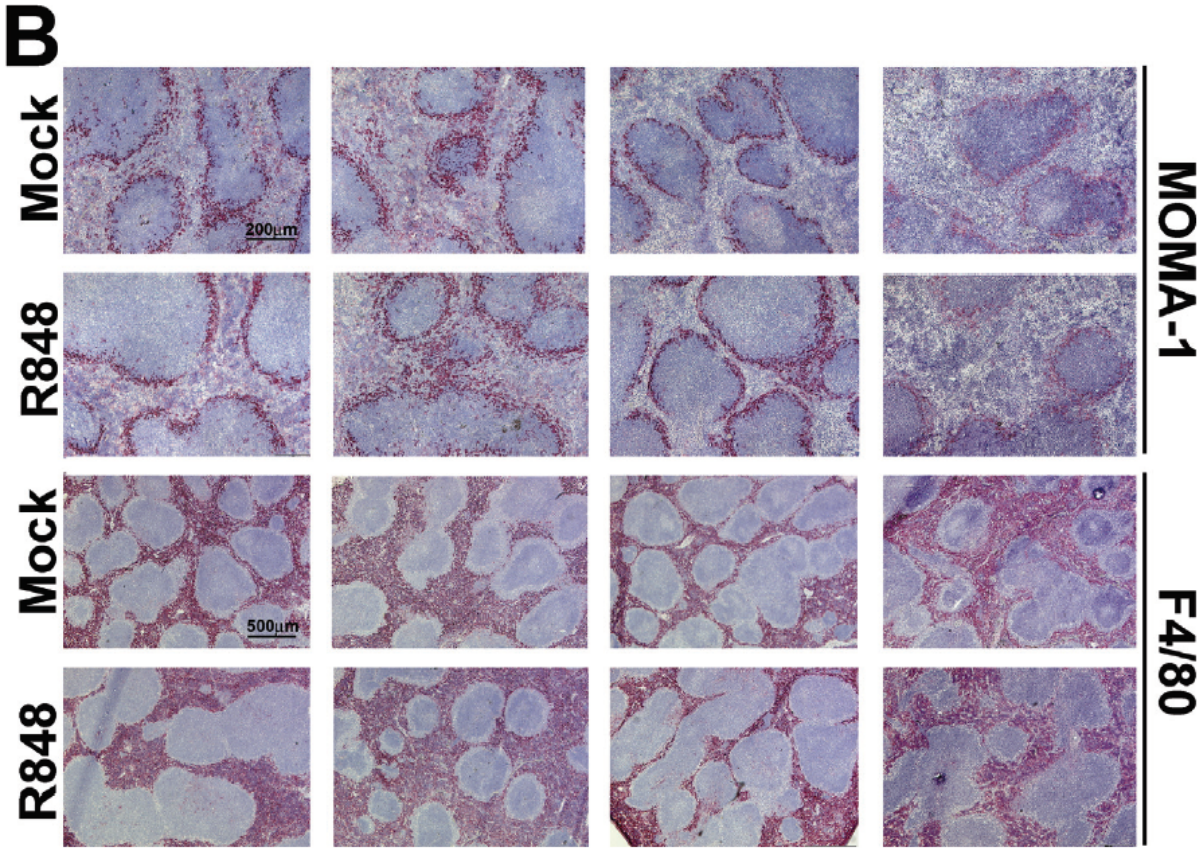

Fig. 7. Disruption of the Lymphoid Structure is TLR7 dependent. (A) Spleen cryosections of C57BL/6, TLR7$^{-/-}$, IFNAR$^{-/-}$ and IRF7$^{-/-}$ mice after 7 days of treatment with 1 mg/kg/d R848, showing a TLR7 dependent malformation of splenic lymphoid follicles since TLR7$^{-/-}$ mice showed normal T- and B-cell zones as well as unaltered marginal zone B-lymphocyte rings. IRF7 knockout mice showed no obvious symptoms of lymphoid follicle malformation. In contrast, IFNAR$^{-/-}$ mice showed somewhat inflated follicles with loosened rings of marginal zone B cells. (B) MOMA-1^{+} and F4/80^{+} macrophages as well as FDC-M1 networks and PNA^{+} germinal centres (data not shown) were not affected. N ≥ 3.

PNA^{+} germinal center B-cell clusters and FDC-M1^{+} follicular dendritic cell-networks were reduced upon treatment for 7 days with 1 mg/kg/d R848 (Fig. 4C). PNA^{+} clusters were increased after 21 and, even more pronounced, after 42 days of treatment with 0.1 mg/kg/d R848, whereas FDC-M1^{+} cell-networks seemed not to be affected. Flow cytometric and RT-PCR analysis confirmed these histological findings: relative frequencies of B220/PNA^{+} and B220/(germinal center B-cell antibody GL7)$^{+}$ splenocytes were reduced after 7 days of TLR7 triggering at 0.1 and 1 mg/kg/d R848, and were increased after 21 and 42 days of treatment at 0.1 mg/kg/d R848 (Fig. 8A). Milk fat globule EGF 8 (MFG-E8) mRNA, a marker for follicular dendritic cells (FDC; Kranich et al., under resubmission), was reduced after 7 days of TLR7 triggering at 1 mg/kg/d, and was unchanged after 21 or 42 days of treatment at 0.1 mg/kg/d (Fig. 8B).

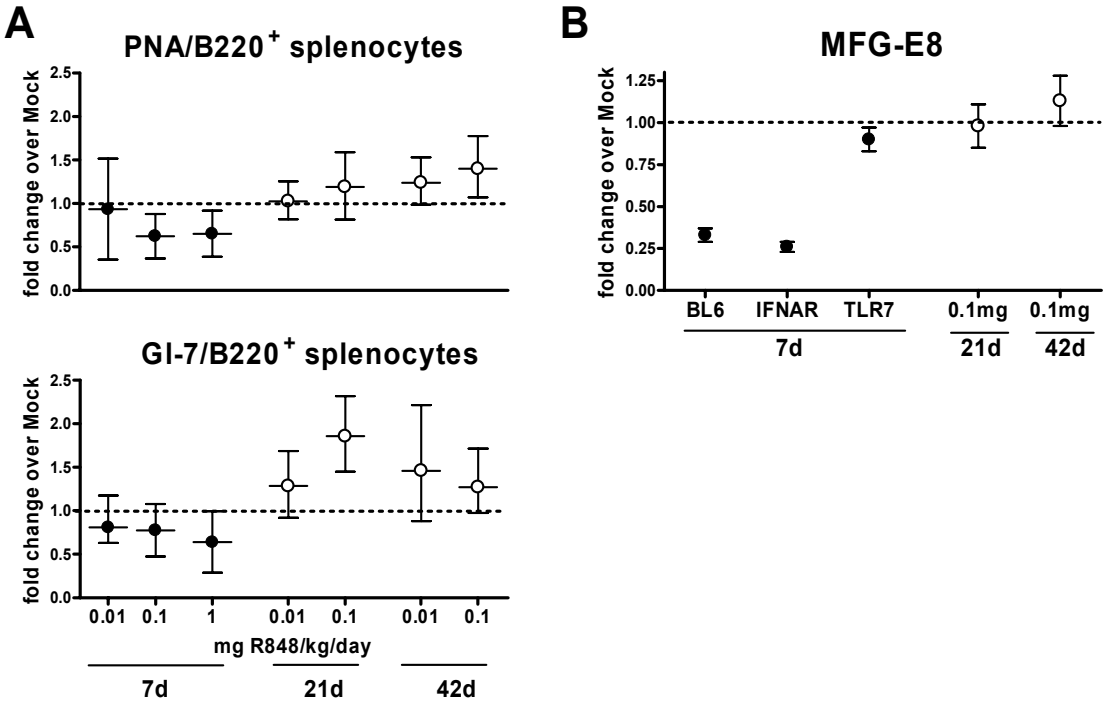

Fig. 8. Germinal center B cells and MFG-E8 mRNA are decreased at day 7 and increased or unaffected at day 21/42 of repetitive TLR7 triggering. (A) C57BL/6 mice were treated as indicated. Splenic germinal center B-cell frequencies were evaluated by flow cytometry by staining for B220 in conjunction with FITC-labeled PNA (upper panel) or GL7 antibody (lower panel). Percentage of double positive cells was normalized to that of corresponding mock treated animals. Error bars indicate 95% confidence intervals. N ≥ 3. (B) RNA was extracted from spleen (n = 3), pooled for reverse transcription and analyzed for MFG-E8 transcripts. Mean normalized MFG-E8 expression was calibrated to the matching mock control. Error bars indicate SEM of mean normalized expression relative to calibrator.

Sustained triggering of TLR7 resulted in an expansion of relative frequencies of myeloid cells and a relative contraction of lymphoid subsets.

An efficient immune response also requires spatially and temporally orchestrated interactions of proper cell types, and this requires organized frequencies and proportions of respective immune cells. We sought to assess splenic immune cell distribution upon triggering TLR7.

After 7 days, spleen weight was 3.3-fold greater in R848-treated than in PBS-treated wild-type mice (Fig. 9A). Notably, although splenomegaly is found in almost 70% of asymptomatic HIV-infected adults [232], it was absent in TLR7$^{-/-}$ mice and less pronounced in IFNAR$^{-/-}$ or IRF7$^{-/-}$ mice after R848 treatment.

Hyperplasia was probably due to myeloid and possibly due to erythroid expansion in the red pulp. Levels of CD11b^{+} macrophages, CD11c^{+} dendritic cells and CD11b^{+}/Gr-1^{+} neutrophils

were increased by about 50% (Fig. 9B upper panel), whereas that of lymphoid subpopulations was decreased. Numbers of $B220^+$ B lymphocytes were reduced by about half as were levels of $CD4^+$ and $CD8^+$ T cells (Fig. 9B, lower panel). Again, reductions were absent in $TLR7^{-/-}$ mice and less pronounced in $IFNAR^{-/-}$ or $IRF7^{-/-}$ mice, indicating that the observed effects depend only in part on IFNAR and IRF7 signalling. We also found a 50% relative reduction of $B220^+$ B lymphocytes in mesenteric lymph nodes, but levels of $CD4^+$ and $CD8^+$ T cells were slightly increased rather than reduced (not shown).

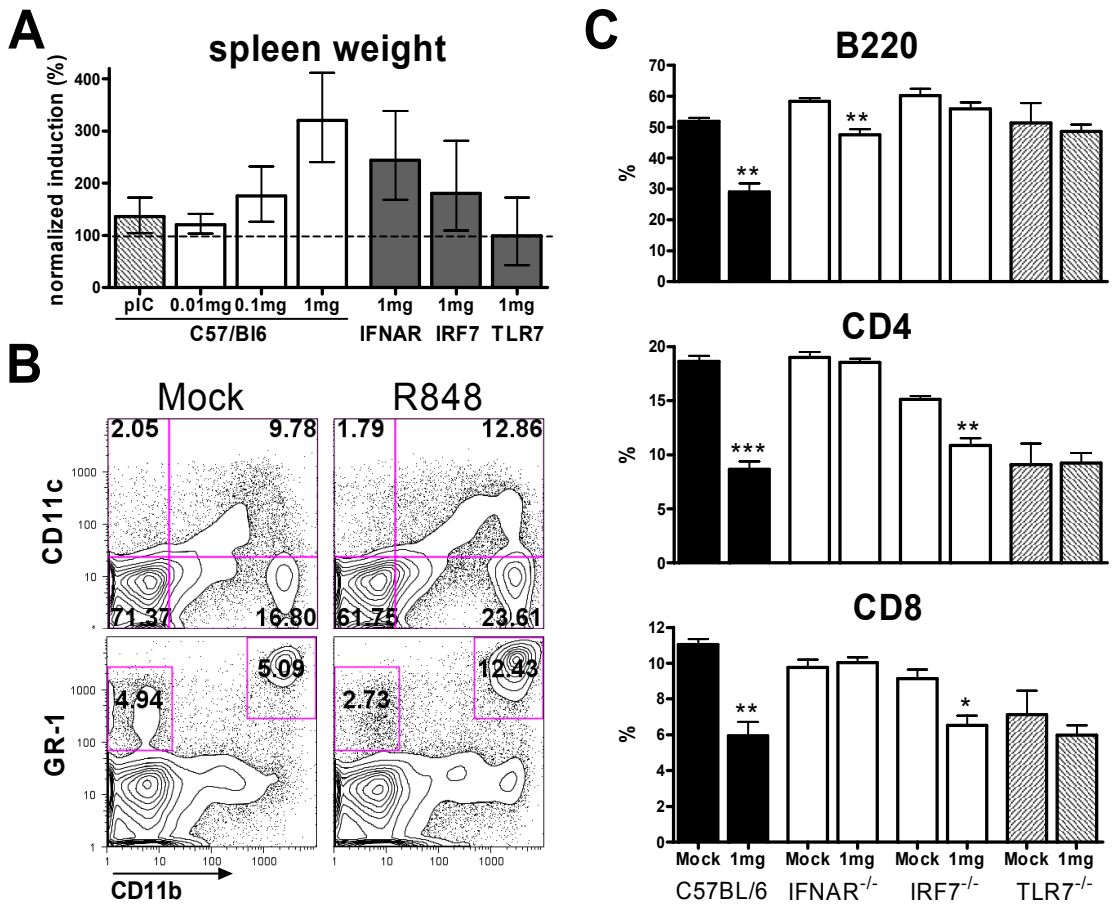

Fig. 9. Splenomegaly is associated with an expansion of relative frequencies of myeloid cells and a relative contraction of lymphoid subsets. (A) Splenomegaly is dose- and TLR7-dependent. Splenomegaly was less pronounced in $INFAR^{-/-}$ or $IRF7^{-/-}$ mice and absent in $TLR7^{-/-}$ mice upon 1 mg/kg/day of R848 treatment. Spleen weight was first normalized to total body weight of each individual mouse and, second, normalized to the spleen weight of mock-treated animals. Error bars indicate 95% confidence intervals. (B) The prevalence of $CD11b^+$ macrophages, $CD11c^+$ dendritic cells and $CD11b^+/Gr\text{-}1^+$ neutrophils was increased, whereas that of lymphoid subpopulations was decreased as compared to mock-treated animals (C). Mice were treated with PBS or 1 mg/kg/day R848 for 7 days. Splenocytes were stained with indicated antibodies and relative numbers were assessed by flow cytometry. Error bars indicate standard error. $N \geq 3$.

Sustained triggering of TLR7 results in rapid and long-lasting cytokine deregulation.

TLR7 activation induces an array of cytokines, including IFNs and proinflammatory cytokines such as TNF-α, IL-6, and IL-12p40. Aberrant cytokine production is a hallmark of HIV infection and may represent a mode by which HIV induces immunodeficiency. Notably, TNF-α, IL-6, and IL-10 levels increase in plasma with kinetics paralleling the raise in plasma viraemia in acute HIV infection and are elevated in chronic infection [233]. Mice treated with 1 mg/kg/d R848 for 7 days were analyzed for plasma cytokine levels in a mouse 23-BioPlex cytokine assay. IL-10, IL-6, TNF-α, and IL-12p40 were the cytokines that were altered to greatest degree (not shown) and therefore investigated in more detail.

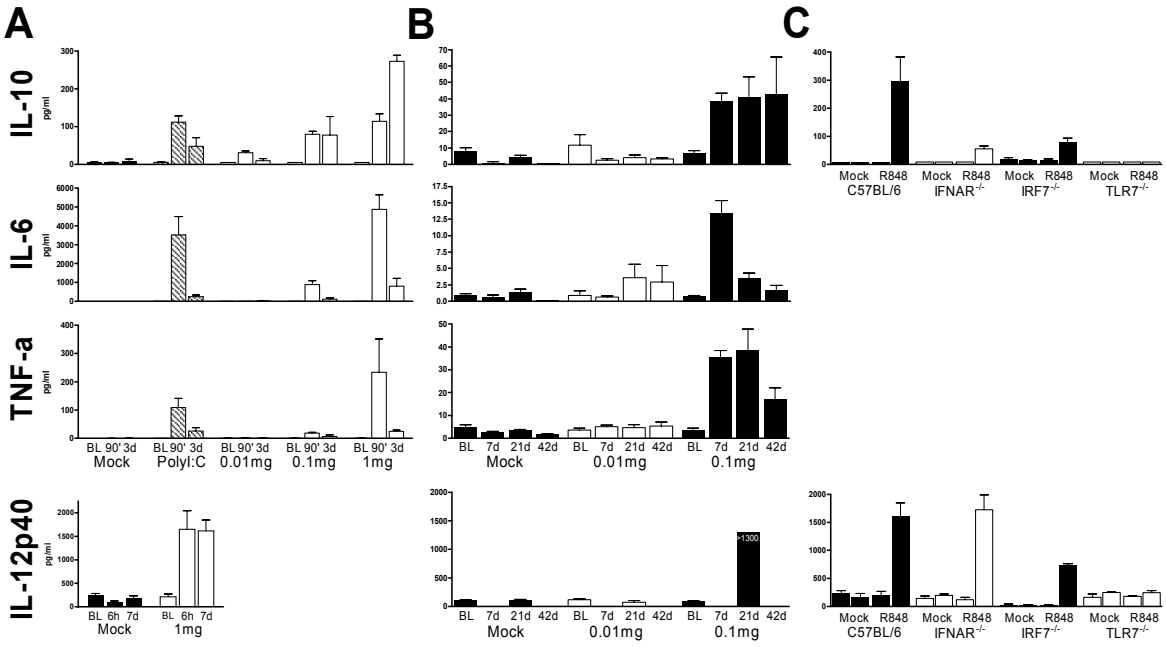

Fig. 10. Rapid and long-lasting cytokine deregulation upon triggering TLR7. Mice were bled from the tail vein, and cytokine levels were measured using a multiplexed particle-based flow cytometric cytokine assay. (A) Rapid cytokine deregulation. Cytokine levels were measured before (BL), 90 min after the first injection, and on day 3 of repetitive treatment. IL-12p40 levels were measured 6 h after a single dose and on day 7 of repetitive treatment. (B) Long-lasting cytokine deregulation. Mice were treated daily at indicated concentrations, and cytokine levels were monitored over time (i.e., on day 7, 21, and 42 of treatment). (C) Cytokine deregulation is TLR7 dependent. C57BL/6 and knockout mice were treated with 1 mg/kg/day of R848 for 7 days. Cytokine levels at day 0 (left bars) and at day 7 (right bars) are shown. Error bars indicate standard error. $N \geq 3$.

Injection of polyI:C or 0.1 and 1 mg/kg/d R848 resulted in an immediate increase of IL-10, IL-6, and TNF-α plasma levels within 90 min, whereas injection of PBS and 0.01 mg/kg/d R848 had no effect (Fig. 10A). Moreover, 1 mg/kg/d R848 resulted in a rapid increase of IL-12p40 as measured 6 h after injection (Fig. 10A). IL-10 and IL-12p40 levels remained high after 3 and 7 daily injections, respectively. IL-6 and TNF-α levels were lower at day 3 than at 90 min, but levels were still higher than in mock-treated mice.

Results obtained after triggering TLR7 over an expanded time period (i.e., up to 42 days; Fig. 10B) with low doses of R848 (i.e., 0.01 and 0.1 mg/kg) were similar to those obtained after triggering TLR7 for 7days (Fig. 10A). Long-term treatment with PBS or 0.01 mg/kg/d R848, again, had no effect. IL-10 and TNF-α were permanently elevated with levels being very stable over time, although TNF-α showed a profound initial burst. IL-6 slowly decreased to almost normal values (Baseline: 0.68 pg/ml, 90 min: 881 pg/ml, 3 d: 114 pg/ml, 7 d: 13.47 pg/ml, 21 d: 3.48 pg/ml, 42 d: 1.62 pg/ml). IL-12p40 consistently was >1300 pg/ml.

R848 treatment (7 days, 1 mg/kg/day) of $IRF7^{-/-}$ or $IFNAR^{-/-}$ mice induced IL-10 and IL-12p40, but to a lesser degree than in C57BL/6 mice (Fig. 10C). In contrast, $TLR7^{-/-}$ mice had normal cytokine levels. In conclusion, the immediate and persistent cytokine deregulation by the action of R848 is TLR7 dependent and reminiscent of HIV infection in humans.

Sustained triggering of TLR7 induces thymic hypocellularity with decreased absolute numbers of CD4 single-positive, CD4/CD8 double-positive, and double-negative thymocytes.

The thymus is the primary lymphoid organ supplying new T lymphocytes to the periphery and might compensate for the loss of T cells caused by HIV. HIV infection, however, results in thymic dysfunction and thymic involution that may recover with potent antiretroviral therapy [234,235]. To investigate whether long-term TLR7 triggering also affects thymic function, we examined thymus cellularity and the percentage of cells in each thymocyte subset by measuring the surface expression of CD4 and CD8 antigens in mice treated with 1 mg/kg/day R848 for 7 days. Total thymocytes were reduced 1.9-fold in R848-treated C57BL/6 mice as compared to mock-treated animals (Fig. 11A). No thymic hypocellularity was detected in $IFNAR^{-/-}$, $IRF7^{-/-}$, or $TLR7^{-/-}$ mice.

Flow cytometric analysis of thymocytes from R848-treated mice revealed an increase in the prevalence of mature $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes from 6.9 to 9.7% and 1.9 to 4.5%, respectively, together with a reciprocal reduction in cells that expressed both CD4 and CD8 (DP) from 79.8 to 72.4% (Fig. 11B). Prevalence of double-negative thymocytes was slightly increased, although not significantly (p=0.11). However, in regard to the 1.9-fold reduction of total thymocytes upon TLR7 activation (Fig. 11A), a different pattern was observed: absolute numbers of all thymocyte subsets were decreased with the exception of $CD8^+$ SP cells. Absolute reduction of DP thymocytes was most prominent because thymuses from R848-treated mice contained less then 50% of DP cells as compared to thymuses from PBS-treated mice.

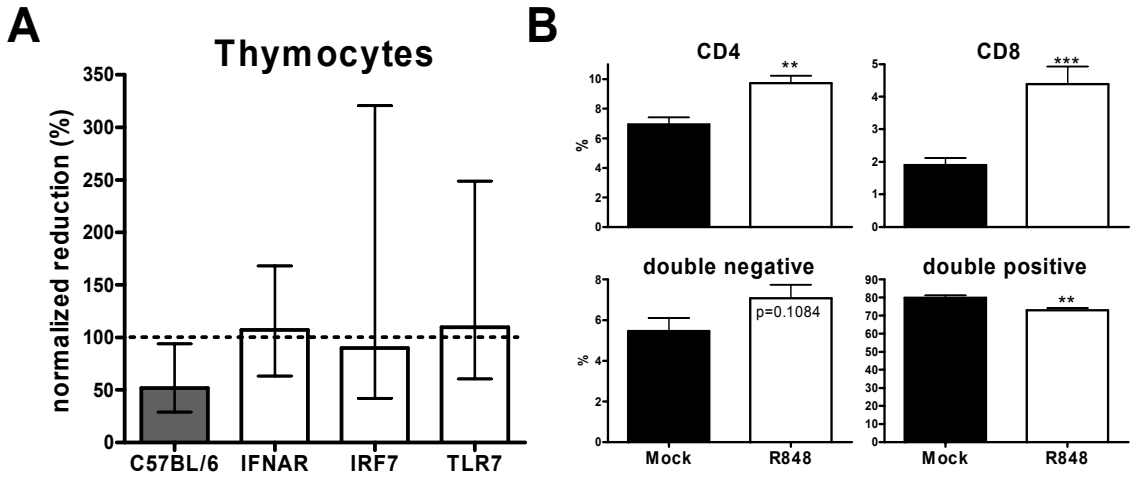

Fig. 11 Thymic hypocellularity upon triggering TLR7. Mice were treated with PBS or 1 mg/kg/day of R848 for 7 days. (A) Thymus was hypocellular in C57BL/6 mice but not in knockout mice upon treatment with R848. Thymocytes were counted manually and normalized to total thymocyte counts of the corresponding mock-treated animals. Error bars indicate 95% confidence intervals. (B) Relative cell numbers in each thymocyte subset were measured in C57BL/6 mice by staining for CD4 and CD8, followed by flow cytometric analysis. Error bars indicate standard error. For C57BL/6 animals data from three independent experiments, each with n = 3, were pooled; n = 3 for knockout mice.

3.3 Discussion

Chronic immune activation is a major cause for progressive immunodeficiency in HIV infection. TLR stimulation is a possible underlying trigger. We used a murine model to assess whether sustained TLR7 stimulation contributes to immune activation by studying the effects on the lymphoid system. Sustained triggering of TLR7 led to i) altered white blood cell counts, including profound lymphopenia and increased neutrophil and monocyte levels ii) immune activation and attenuated humoral immune responses, iii) disruption of the lymphoid structure, including splenomegaly, enlarged T and B cell zones, reduced marginal zone B-lymphocytes and a relative contraction of lymphoid subsets, iv) cytokine deregulation, v) thymic hypocellularity. We also showed that the observed effects were TLR7- and dose dependent, and that IRF-7 and IFNAR mediated signalling only partly contributed to R848 mediated pathology.

Sustained TLR7 triggering led to altered white blood cell counts. We first monitored blood parameters, because hematological abnormalities are common in HIV-infected patients. Repetitive TLR7 triggering resulted in a depletion of lymphocytes from the blood together with a reciprocal increase of neutrophils and monocytes. This effect was dose dependent and, more importantly, stable as long as R848 treatment was maintained. In fact, TLR7 stimulation did not lead to steadily increasing amounts of damage or to an attenuated response over time, pointing to the possibility that the level of TLR7 signalling over time is a major determinant of the observed effects. Of note, individuals with higher HIV RNA levels 6 months after seroconversion had faster progression to AIDS [236]. Blood from R848-treated mice also showed a tendency towards lower thrombocyte counts. In HIV infection, the number of platelets can be low [237] due to trapping of platelets in the enlarged spleen or abnormally high levels of TNF-α [238], which is released upon TLR7 engagement. Two recent studies investigated lymphopenia in acute viral infection and in treatment with a single systemic dose of immunomodulatory agents, such as TLR ligands [225,226]. Our results revealed two net differences: i) lymphocytes but not granulocytes or monocytes were depleted from the blood by R848, and ii) lymphopenia was rather specific to TLR7 activation than an universal phenomenon of TLR triggering, since triggering TLR3 had no effect. The latter may be explained by different doses that were applied in the different studies. The facts that TLR3 and TLR7 activation both induced similar cytokine patterns, but only TLR7 activation induced lymphopenia, also indicate that chronic activation of distinct TLRs over time results in characteristic lesions.

Sustained TLR7 triggering led to immune activation and attenuated humoral immune responses. R848-induced lymphopenia with its concurrent reduction of immune effector cells might lead to immune insufficiency and increased susceptibility to infections [225]. On the other hand, R848 is currently discussed as an effective vaccine adjuvant to stimulate humoral immune responses [239,240] since TLR7 ligands directly stimulate B cells [241]. Our observation that sustained TLR7 triggering led to immunosuppression with weakened antigen-specific immune responses suggests that sustained TLR7 stimulation may be harmful while temporary and time-limited triggering may result in antiviral and protective effects. This is in line with a previous report, where guinea pigs infected with Herpes simplex virus showed impaired antibody responses upon repetitive TLR7 triggering [242].

The findings that R848 treatment stimulated B cells to express an activated phenotype and secrete unspecific antibodies indicated that immunosuppression was not due to a general incapability of B cells to produce IgG. B-cell activation and hypergammaglobulinaemia are characteristic for HIV infection [228,230].

Sustained TLR7 triggering led to disruption of the lymphoid structure. Secondary lymphoid organs are the critical place of HIV replication *in vivo*, even shortly after infection, when HIV expression is active throughout the period of asymptomatic HIV infection [161]. Hence the ssRNA burden in lymphoid tissue may perpetually trigger TLR7 from the early time point of infection. We thus tested whether TLR7-mediated changes in spleen morphology and composition accounted for the weakened antigen-specific humoral immune response. Indeed, the prevalence and density of splenic lymphoid subsets (i.e., $CD4^+$, $CD8^+$, and $B220^+$ cells) decreased upon R848 treatment, probably due to disproportionate myeloid expansion reducing the efficiency of immune cells to exert cell-cell contact mediated functions and very likely underlies the observed effect of immunosuppression. We also show that sustained TLR7 triggering caused splenomegaly, follicular hyperplasia, T- and B-cell zones enlargement, and a loss of marginal zone B lymphocytes, further emphasizing that sustained TLR7 triggering may contribute to disease progression in HIV infection. Notably, splenomegaly is often found in HIV-infected individuals [232] and follicular hyperplasia has been reported in early-stage disease [161].

As HIV disease progresses, there is a shift from follicular hyperplasia to follicular involution with disruption of the FDC network being characteristic during this transition [243]. Upon R848 treatment for 7 days, FDC networks were reduced. At day 21 or 42, however, FDC networks seemed not to be affected. Involution might occur after much longer periods of TLR

triggering or if higher doses of R848 would be applied. Nevertheless, the R848-induced hyperplasia and the loss of intact lymphoid tissue architecture may give way to follicular involution, fibrosis, and even lymphocyte depletion. Remarkably, our data also fit with data from a study in transgenic mice with the systemic lupus erythmatosus susceptibility 1 (*Sle1*) locus and a translocated copy of TLR7, which results in a twofold TLR7 expression. These mice develop pronounced splenomegaly, increased numbers of activated T cells, and aberrant expansion of most immune cell lineages, especially monocytes and neutrophils [244,245].

Sustained TLR7 triggering led to cytokine deregulation. TLR7 triggering induced permanently elevated IL-10, IL-6 , and TNF-α levels, which also are elevated in chronic HIV infection [233]. IL-10 may have a crucial role in immuoregulation by balancing pathogen-specific immunity and immune-mediated tissue damage. Remarkably, in lupus-prone mice, abnormal TLR9-mediated IL-10 cytokine production could account for a number of immune abnormalities, such as increased B-cell survival, chronic activation, and hypergammaglobulinaemia [246]. Observations from LCMV models have also suggested that IL-10 blocking strategy may positively affect chronic viral infections, such as HIV or hepatitis C virus (HCV) in humans [247-249]. Similarly, polymorphisms that reduce IL-10 expression have been associated with slower progression to AIDS [101]. Finally, IFN-α and ribavirin, the current therapy for HCV, downmodulate IL-10 secretion [250]. Because IL-6 plays an essential role in the differentiation of activated B cells, IL-6 overproduction may contribute to polyclonal B-cell activation and to the pathogenesis of AIDS by inducing HIV replication [251].

Chronic, low-level TNF expression causes profound disturbances in tissue development, especially a diminution of thymic tissue [99]. In line with these data, R848-treated mice showed thymic hypocellularity, indicating that TLR7 stimulation may damage thymic function and thereby impair the thymus' capacity to compensate for the loss of T cells caused by HIV. Taken together, these data imply that TLR7-mediated immunoregulatory cytokine deregulation can influence the outcome of chronic infections. Their modulation could be used as a therapeutic strategy to improve the outcome for the host.

IRF-7 and IFNAR mediated signalling only partly contributed to R848 mediated pathology. Using mice deficient for $TLR7^{-/-}$, we clearly show that R848-mediated effects were TLR7 dependent. Upon ligand recognition, TLR7 triggers innate immune responses and IFNs through NF-κB- and IRF7-dependent signalling pathways, respectively [252]. Recent reports demonstrated that IRF7 is essential for the induction of IFN-α/β genes via the TLR-activated

MyD88-dependent pathway [253], and that IFN-α/β directly regulate lymphocyte recirculation and cause transient blood lymphopenia [226], pointing to a potential role of IFNAR signalling in R848-induced pathology. Although effects upon TLR7 triggering in $IFNAR^{-/-}$ or $IRF^{-/-}$ mice were generally less pronounced, they were still quite impressive (e.g., lymphopenia). This was unexpected since lymphopenia is critically dependent on IFNAR signalling [226]. Also counterintuitively, IFN-α levels were not elevated upon repetitive R848 treatment (not shown). However, in the above-mentioned study mice were given a single dose of R848, while in our study, mice were treated for several days. Daily dosing with high doses of TLR7 ligands for 5 consecutive days led to tachyphylaxis: IFNs were almost completely down-regulated [254], which is normally not seen when doses are separated by more than 2 days. Taken together, these data imply that short- and long-term TLR7 activation may have different mechanisms of action. IFNAR and IRF7 signalling only partly contributed to R848-mediated pathology, but other pathways may also be involved, possibly through NF-κB (e.g., IL-6, IL-10, TNF induction).

In conclusion, our data demonstrate that sustained TLR7 triggering in mice results in immune activation and disruption of the lymphoid system reminiscent of HIV-associated pathology. This underlines that HIV ssRNA itself probably contributes to persistent immune activation and pathology by signalling through TLR7 and explains in part the chronic immune dysfunction in HIV infection. Hence, manipulating TLR7 triggering or down-stream signalling may be therapeutically valuable to reduce chronic hyper-immune activation and immune dysfunction.

3.4 Material and Methods

Mice and Treatment

C57BL/6 mice were purchased from Harlan (The Netherlands). IFNAR$^{-/-}$ and TLR7$^{-/-}$ mice were kindly provided by Karl S. Lang (Institute of Experimental Immunology, University Hospital of Zurich, Zurich, Switzerland). IRF7$^{-/-}$ mice were provided by Kenya Honda (University of Tokyo, Tokyo, Japan). Knockout status of all animals was verified by PCR analysis with DNA isolated from the tail. C57BL/6, IFNAR$^{-/-}$, and IRF7$^{-/-}$ mice were housed under specific pathogen-free conditions. TLR7$^{-/-}$ animals were housed in filter cages under conventional conditions. All procedures were approved by the veterinarian authorities from the Canton of Zurich, Switzerland.

Age- and sex-matched mice (6–8 weeks) were injected intraperitoneally (i.p.) on a daily basis for 7 days with 0.01, 0.1, or 1 mg/kg/day R848 and for 21 or 42 days with 0.01 or 0.1 mg/kg/day R848. Control mice were treated with polyI:C (50 μg/mouse/day) or with PBS. R848 was kindly provided by 3M Pharmaceuticals. 1 mg/kg/day R848 amounts to a dose of 20 μg per injection. Polyinosinic-polycytidylic acid (polyI:C) was from Sigma. Substances were dissolved in PBS and administered i.p. at 200 μl/dose. Mice were analyzed 1 h after the last injection unless otherwise stated.

Quantification of Peripheral Blood Leukocytes

Blood samples were collected in heparinized microtainers (BD Biosciences) by terminal bleeding from the heart 1–2 h after the last R848 injection. Absolute and relative numbers of leukocytes, lymphocytes, monocytes, neutrophils, and thrombocytes were quantified on an Abbott Zelldyn 3500 or counted manually after staining on Hematec (Bayer) at the veterinarian-medical hospital, Zurich, Switzerland.

Flow Cytometry

Absolute counts of lymphocyte subpopulations were determined by BD truCOUNT technology and by staining the samples for CD4 (clone L3T4), CD8 (Ly-2), and B220 (RA3-6B2), according to the manufacturer's protocol. The activation status of the splenocytes was analyzed by four-color flow cytometry with monoclonal antibodies (mAbs) against CD4, CD8, B220, and CD69 (H1.2F3) after lysis of erythrocytes with ACK lysis buffer. Similarly, frequencies of myeloid cells in spleen were determined by staining for CD11b (M1/70), CD11c (HL3), and/or Gr-1 (RB6-8C5). Splenic germinal center B-cell frequencies were

evaluated by staining for B220 in conjunction with FITC-labeled peanut agglutinin (PNA; Vector Laboratories) or GL7 antibody. All antibodies were from BD PharMingen. Cells were acquired on a FACS Calibur (BD Biosciences). Data were analyzed with FlowJo 7.1 software.

VSV-specific Serum Neutralization Test and Determination of Total IgG

The Indiana strain of VSV (VSV-IND; Mudd-Summers isolate) was originally obtained from D. Kolakofsky (University of Geneva, Geneva, Switzerland), propagated on BHK-21 cells, and allowed to form plaques on Vero cells. Before injection in mice, VSV-IND was ultraviolet-inactivated (UV Stratalinker 1800 with 5 UV light bulbs of each 8W (254 nm); Stratagene) for 5 min in an open 60-mm Petri dish.

To measure neutralizing antibody titers, sera were prediluted 16-fold in supplemented Dulbecco's Modified Eagle Medium (DMEM) and heat-inactivated for 30 min at 56 °C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 PFU/ml. The mixture was incubated at 37 °C and 5% CO_2 for 90 min. The serum-virus mixture (100 µl) was transferred onto Vero cell monolayers in 96-well plates and incubated at 37 °C for 1 h. Monolayers were overlaid with 100 µl DMEM containing 1% methylcellulose. After incubation at 37 °C for 24 h, the overlay was removed, and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the neutralizing titer 50. To determine IgG titers, undiluted serum was pretreated with an equal volume of 100 mM β-mercaptoethanol in saline. Total IgG was quantified with mouse IgG ELISA quantification kit (Bethyl Laboratories), according to the manufacturer's instructions.

Histology and Immunohistochemistry

Spleens were stored in Hank's Buffered Salt Solution (HBSS) and flash frozen in liquid nitrogen for immunohistological analysis. Frozen sections of the spleen (6 µm) were stained with H&E. Antibodies FDC-M1 (4C11; 1:50, Becton Dickinson) and CD21/35 (8C12; 1:100; PharMingen) and antibodies to CD45RO/B220 (RA3-6B2; 1:400; PharMingen), MOMA-1 (1:50; BMA; Augst), F4/80 (1:50; Serotec), PNA (1:100; Vector Laboratories), CD4 (YTS 191; 1:200), and CD8 (YTS 169; 1:50) were used as primary reagents for immunohistochemistry and lectin histochemistry, as described [255]. Antibodies against CD4 and CD8 were kindly provided by R. Zinkernagel (University Hospital of Zurich, Zurich, Switzerland). Image acquisition was performed on a Axiophot-microscope (Zeiss) equipped with a JVC digital camera (KY-F70; 3CCD) and analyzed with their software.

Measurement of Serum Cytokines

Mouse blood was collected into serum separator microtubes (BD Biosciences) by tail-vain bleeding or cardiac punctuation and centrifuged for 10 min at 5,400 g to obtain serum. Cytokine protein levels in serum were measured using a multiplexed particle-based flow cytometric cytokine assay. Bioplex mouse kits for IL-6, IL-10, IL-12 (p40), and TNF-α were purchased from BioRad. The procedures closely followed the manufacturer's instructions. The analysis was conducted using FC 500 flow cytometer (BeckmanCoulter).

PCR Array Profiling and MFG-E8 RT-PCR

Splenic samples were immediately subjected to RNAlater (Qiagen) and stored at -20 °C until use. RNA was extracted from 20 mg of samples with the RNeasy mini-kit (Qiagen) according to the manufacturer's instructions. For tissue homogenates, blocks were disrupted with a mortar and pestle in lysis buffer containing 2-ME. After extraction, RNA was pooled from replicates (n = 3). To remove residual DNA, RNA preparations were treated with DNA*free* (Ambion) according to the manufacturer's instructions. RNA quality, as judged by gel electrophoresis and spectrophotometry, was comparable in all samples. Reverse transcription was performed with 5 µg of total RNA in a total volume of 50 µl. RNA was incubated with 1250 ng of oligo(dT) primer (Life Technologies) and 500 µM dNTPs (Roche) at 65°C for 5 min, followed by incubation on ice for 5 min. Samples were then incubated with 3 mM $MgCl_2$ (Promega) and 60 U of RNase inhibitor (Roche) in 1x ImProm-II RT reaction buffer (Promega) at 42°C for 2 min. ImProm-II RT (500 U; Promega) was added, and reactions were performed at 42°C for 50 min, followed by inactivation at 65°C for 15 min. Residual RNA was digested with 1250 ng of RNase (Roche) at 37°C for 20 min.

RT-PCR was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems) with conditions as mentioned above. MFG-E8 mRNA, representative of follicular dendritic cell marker FDC-M1, and GAPDH housekeeping gene were detected using ready-to-use primer/probe kits (Applied Biosystems; Mm00500549_m1 and Mm99999915_g1). All reactions were performed in triplicate. Data were analyzed in two steps. First, the mean normalized gene expression (MNE) for every sample was determined using the software application Q-Gene (calculation procedure for MNE 2) [256]. Second, normalized MFG-E8 expression, relative to that of respective Mock treated mice was assessed.

Statistical Analyses

Data are depicted as means and SEM. Means were compared using the unpaired *t* test, unless otherwise stated in the figure legends (e.g., * $p<0.05$, ** $p<0.01$, *** $p<0.001$). 95% confidence intervals of normalized data were computed using graph pad software, according to EC Fieller [257].

3.5 Acknowledgements

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4

Impact of CD4/CCR5 signalling on HIV replication

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4. CD4 but not CCR5 Signalling Affects Early HIV Replication

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HIV infects cells through a receptor complex consisting of the CD4 receptor and one of the chemokine receptors CCR5 or CXCR4. Each component of the receptor complex is involved in signal transductions, raising the question of whether HIV exploits them to optimize conditions for viral replication. Here we analyzed several steps of the HIV replication cycle of two CCR5-tropic (R5) HIV molecular clones and three R5 patient isolates in a panel of cell lines stably expressing different combinations of either wildtype (wt) or mutant CD4 (wt or 398) and CCR5 (wt, R126N, 320, or double mutant). Phenotypical and functional analyses of the cells demonstrated intact and deficient signalling in the wt and mutant cell lines, respectively. Quantities of early reverse transcripts in 398/wt and 398/320 cells were enhanced relative to those in wt/wt cells, suggesting that disruption of signalling through CD4 increased the efficiency of early reverse transcription. There was no statistically significant difference in the amount of early reverse transcripts between 398/wt and 398/320 cells, indicating that signalling through CCR5 does not have a major effect on early HIV replication. In contrast to early reverse transcription, levels of productive infection and virus production did not appear to be directly affected by disruption of CD4 and/or CCR5 signalling. In conclusion, our data suggest that i) signalling through CD4 has an inhibitory effect on early reverse transcription, whereas CCR5 signalling does not play a major role, and ii) later steps of the HIV replication cycle are not modulated by CD4/CCR5 signalling.

4.1 Introduction

Entry of HIV-1 into cells requires interactions between the HIV envelope glycoprotein (gp)120 and a cellular receptor complex consisting of the CD4 receptor and one of the chemokine receptors CCR5 or CXCR4 [258]. HIV isolates are classified based on their chemokine co-receptor use into CCR5-tropic (R5) and CXCR4-tropic strains [135]. R5 isolates are considered more relevant in HIV pathogenesis because they are responsible for viral transmission, predominate during the early stages of the disease, and are present throughout the whole infection period [259]. Individuals homozygous for a 32–base pair deletion in the coding region of CCR5 are almost completely resistant to HIV infection [260,261].

Each component of the receptor complex is also involved in signal transduction networks. CD4 serves as an accessory molecule of the TCR/CD3 complex for binding peptide-MHC complexes [262]. Its cytoplasmic tail noncovalently associates with the lymphocyte-specific protein tyrosine kinase p56lck (Lck) [122,263-267]. Cross-linking of CD4 with antibodies or the binding of peptide-MHC complexes or IL-16 to CD4 results in phosphorylation of Lck [268-270] and subsequent $CD4^+$ T-cell activation [270-272].

The two chemokine receptors and their ligands are key regulators of leukocyte trafficking to inflamed tissues [273]. Natural ligands for CCR5 include RANTES, MIP-1α, and MIP-1β. CCR5 belongs to the family of seven-transmembrane domain receptors that signal through heterotrimeric G-proteins upon ligand binding. Chemokine receptors have several conserved structural features, including a DRY motif in the second intracellular loop, which is involved in G-protein interaction, and structural motifs in the cytoplasmic tail, which are critical for signalling, desensitization, and receptor trafficking [274].

The binding of HIV-1 gp120 to CD4 or the chemokine co-receptor also initiates signals, some of which are specific for HIV, such as the activation of non-selective cation channels [275]. Signalling through CD4 or CCR5 does not appear to affect HIV-1 entry [147,149,276-279]. In contrast, post-entry steps of the HIV replication cycle are modulated by CD4-mediated signalling, but it is unclear whether CD4 negatively [146] or positively [147-151] regulates HIV replication. Similarly, the effects of signalling through the chemokine co-receptor are a matter of debate: for example, one study found CCR5-mediated signalling to be dispensable for HIV replication [280], whereas another showed that CCR5 signalling facilitates HIV replication [281].

Previous studies focused on signalling by the components individually. However, the two receptors are physically [282] and functionally [283-285] associated. Here, we generated human T-

cell lines stably expressing different combinations of either wild-type (wt) or mutant CD4 and CCR5. These cell lines were used to study both the overall influence of signalling through the receptor complex on HIV replication and the contribution of each receptor to such effects. The mutations in CD4 and CCR5 were chosen based on motifs known to be involved in signal transductions. The cells lines were inoculated with two R5 molecular clones and three R5 patient isolates and the efficiency of different stages of HIV replication in the mutant cell lines was compared to that in the wt CD4/CCR5 cell line.

4.2 Results

Receptor expression and proliferation of T-cell lines stably expressing wt or mutant CD4 and CCR5.

To assess the effects of signalling through CD4 and/or CCR5 on HIV replication, we generated T-cell lines stably expressing different combinations of wt or mutant CD4 and CCR5 (Fig. 1) by means of lentiviral transduction. The mutations introduced into CD4 and/or CCR5 (i.e., deletion of the cytoplasmic tail of CD4; single mutation of the DRY motif in CCR5, partial deletion of the cytoplasmic tail of CCR5, or both) were chosen based on motifs known to be involved in signal transductions. For example, signalling through CD4 is mediated by the interaction of p56lck with the cytoplasmic tail of CD4 [263]; the DRY motif of CCR5 is involved in G-protein interaction, and structural motifs in the cytoplasmic tail are critical for signalling, desensitization and receptor trafficking [274]. These cell lines were derived from A2.01 cells, a $CD4^-$ $CCR5^-$ derivative of the human T-cell leukemic line, A3.01 [286]. The cell lines will be referred to by abbreviations that indicate the variant of CD4/CCR5 expressed.

Cell surface expression of CD4 and CCR5 was analyzed after several weeks of selection. CD4.398 was expressed at lower levels than those of CD4.wt (Fig. 2A). For each CCR5 variant, expression was similar in cells co-expressing either CD4.wt or CD4.398 (Fig. 2B). CCR5.wt and CCR5.R126N were expressed at fairly high levels, whereas CCR5.320 and especially CCR5.dm showed lower expression levels. Notably, receptor expression levels were reproducible in different transduction experiments and stable over time (data not shown).

Cell proliferation was similar in all cell lines as assessed by flow cytometry of cells stained with CFSE, showing that the mutations introduced into CD4 and/or CCR5 had no adverse effect on cell proliferation (Fig. 3).

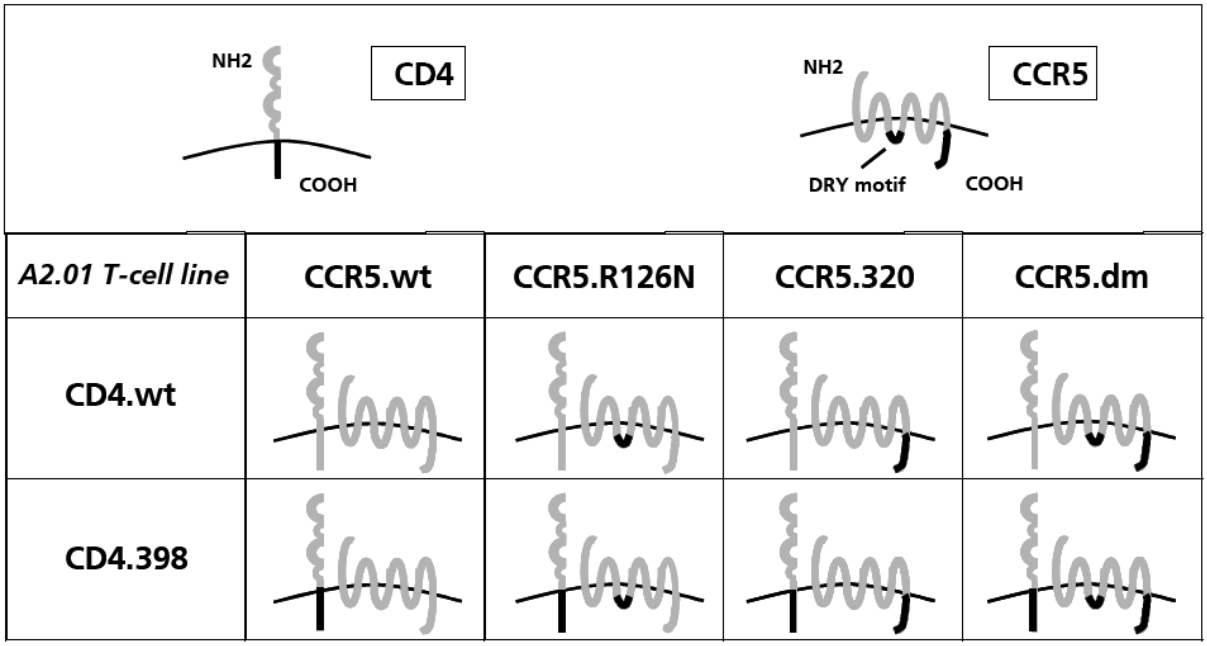

Fig. 1. Schematic representation of A2.01 T-cell lines expressing different combinations of wt or mutant CD4 and CCR5. A2.01 cells were first genetically complemented with wild-type CD4 (CD4.wt) or CD4 with a deletion of the cytoplasmic tail after residue 398 (CD4.398). Subsequently, these cell lines were additionally complemented with wild-type CCR5 (CCR5.wt) or CCR5 with a point mutation in the DRY motif (CCR5.R126N), a deletion of the cytoplasmic tail after residue 320 (CCR5.320), or both mutations (double mutant; CCR5.dm). The cell lines will be abbreviated as follows: wt/wt, wt/R126N, wt/320, wt/dm, and 398/wt, 398/R126N, 398/320, 398/dm.

Disruption of signal transduction pathways through CD4 and/or CCR5 mutations.

Stimulation of $CD4^+$ T cells with antigen results in phosphorylation and internalization of CD4, and this response can be mimicked by treatment with phorbol esters [287]. CD4 down-modulation in response to phorbol esters is abrogated if the cytoplasmic tail of CD4 is removed [147]. Indeed, CD4.wt but not CD4.398, was down-modulated upon stimulation of the cells with PMA, irrespective of which CCR5 variant was co-expressed (Fig. 4), showing that CD4.wt, but not CD4.398 was responsive to stimuli.

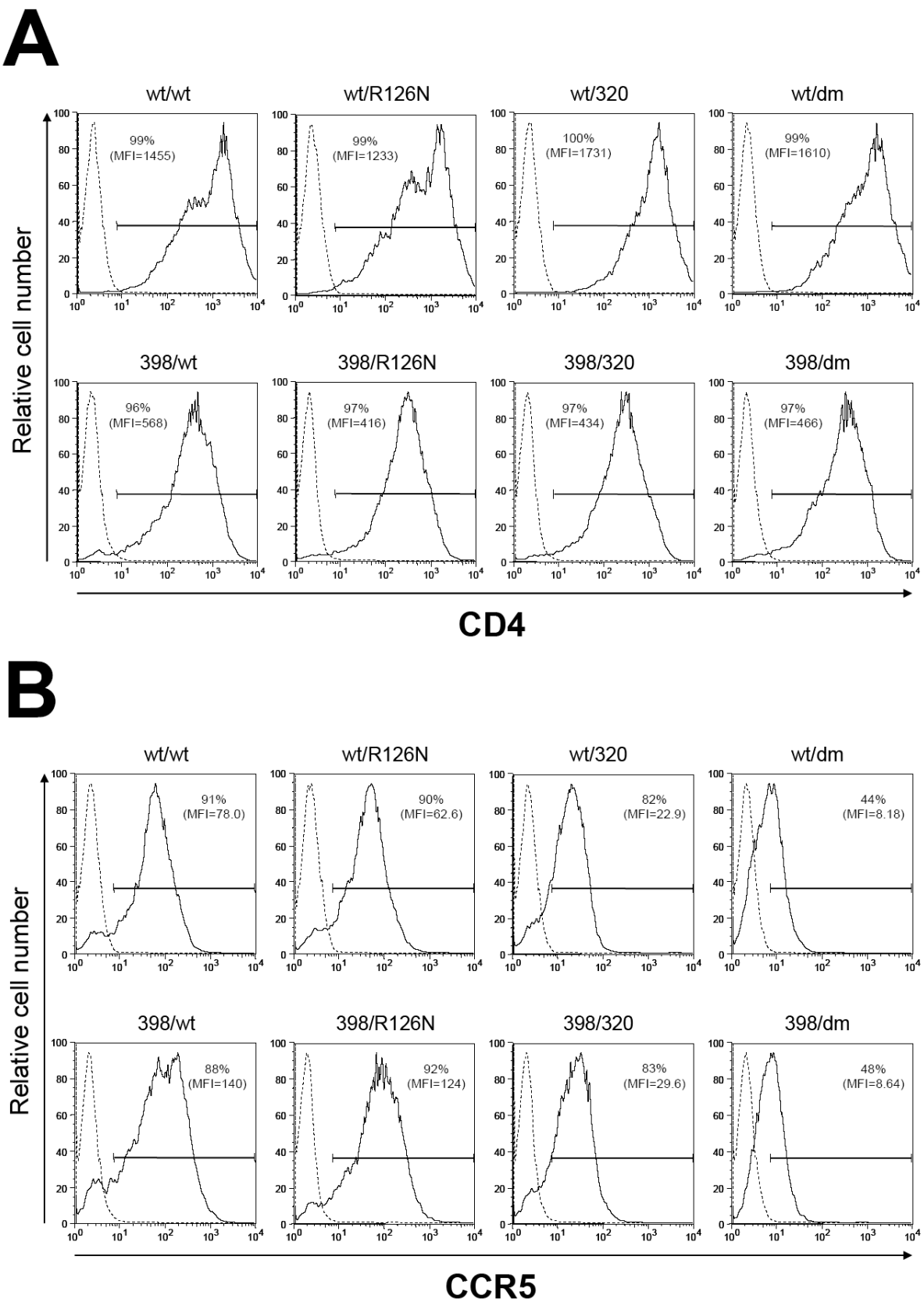

Fig. 2. Cell surface expression of CD4 and CCR5 in CD4/CCR5-expressing A2.01 T-cell lines. After several weeks of selection, cells were stained with a mAb against either CD4 (A) or CCR5 (B). Receptor expression was analyzed by flow cytometry. Dashed and solid lines indicate cells stained with isotype or specific mAb, respectively; MFI = mean fluorescence intensity.

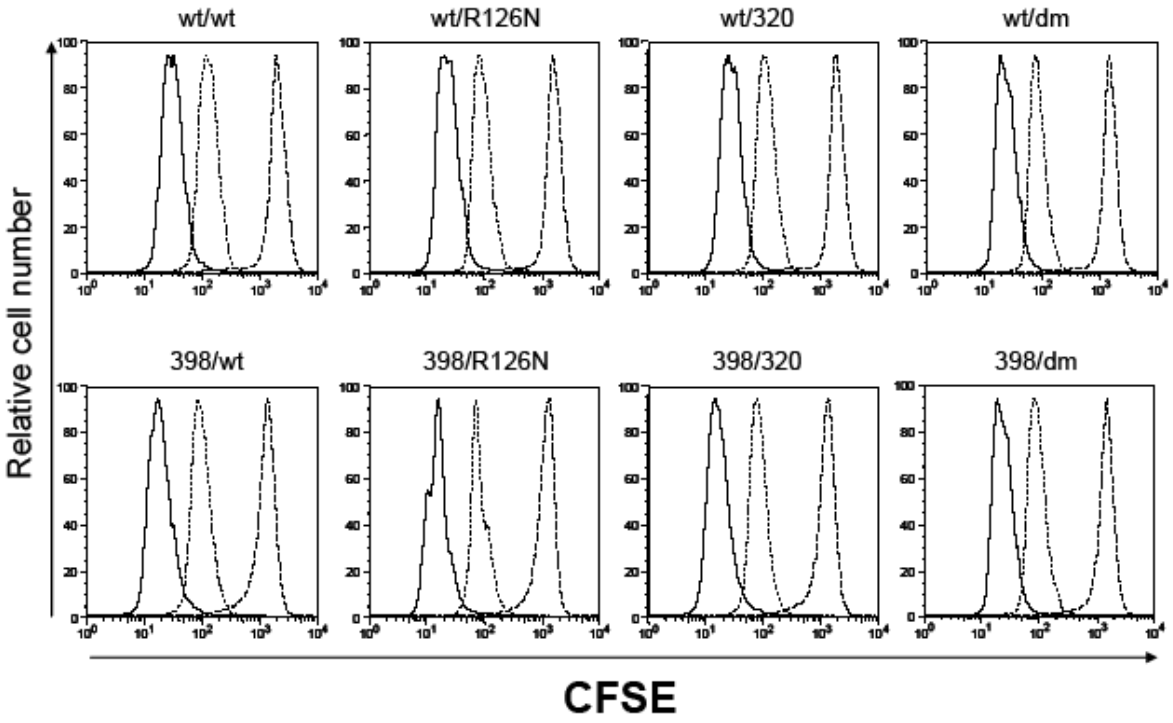

Fig. 3. Cell proliferation of A2.01 T-cells expressing different combinations of wt or mutant CD4 and CCR5. Cells were stained with CFSE, and aliquots of cells were analyzed by flow cytometry immediately (broken lines), one day (dashed lines) or four days (solid lines) after staining. Results are representative of three experiments.

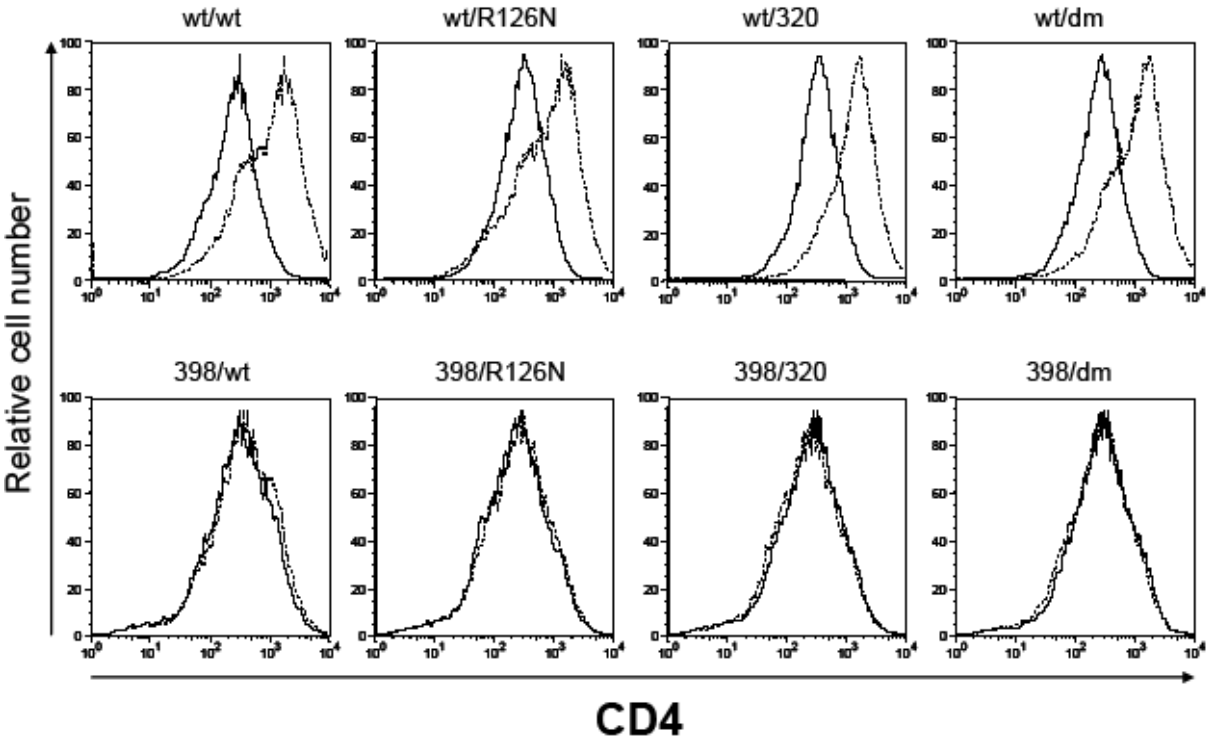

Fig. 4. Effect of CD4 mutation on signalling. Cells were stimulated with PMA (0.05 μg/ml; solid lines) or not (dashed lines). After 4 h, cells were analyzed for CD4 cell surface expression by flow cytometry. Results are representative of four experiments.

CCR5 variants were functionally characterized by analyzing ligand-stimulated receptor endocytosis/recycling and signalling. As reported [288,289], CCR5.wt, CCR5.R126N, and CCR5.320 were internalized with similar efficiencies in response to MIP-1β (Fig. 5A, left panel). In contrast, much less CCR5.dm than wt receptor was internalized. When the ligand was withdrawn, all CCR5 variants completely recovered (Fig. 5A, right panel). These results were independent of expression of CD4.wt or CD4.398. To corroborate these data, we also analyzed MIP-1β-induced CCR5 internalization with acid washes [289] to remove residual surface-bound chemokine. This protocol gave similar results (data not shown).

Stimulation of cells with MIP-1β consistently resulted in actin polymerization in cells expressing CCR5.wt or CCR5.320 as assessed by flow cytometric detection of phalloidin-$FITC^+$ cells (Fig. 5B). Phalloidin binds to polymerized actin. The response was higher in cells co-expressing CD4.398 than in those co-expressing CD4.wt. Actin polymerization was slightly lower in wt/320 and 398/320 cells than in wt/wt and 398/wt cells. Wt/R126N and 398/R126N cells showed no actin polymerization, whereas the response in wt/dm and 398/dm cells was variable. Similar results were obtained when MIP-1β-induced chemotaxis was analyzed (data not shown).

As reported [276,277,280,281,288], mobilization of intracellular calcium in response to MIP-1β was abrogated in cells expressing CCR5.R126N or CCR5.dm and slightly reduced and prolonged in cells expressing CCR5.320 (Fig. 5C). As observed with actin polymerization, cells co-expressing CD4.398 showed a stronger MIP-1β-induced increase of intracellular calcium concentrations than those co-expressing CD4.wt. This finding is consistent with data showing a reciprocal cross-desensitization between the two receptors [284], indicating CD4/CCR5 cross-talk.

These results reinforce our concept of investigating CD4/CCR5 receptors as an entity. To summarize, functional characterization of our cell lines confirmed disruption of signal transduction pathways through CD4 and CCR5 in the mutant cell lines, validating our experimental system to investigate the role of CD4/CCR5-mediated signalling on HIV replication.

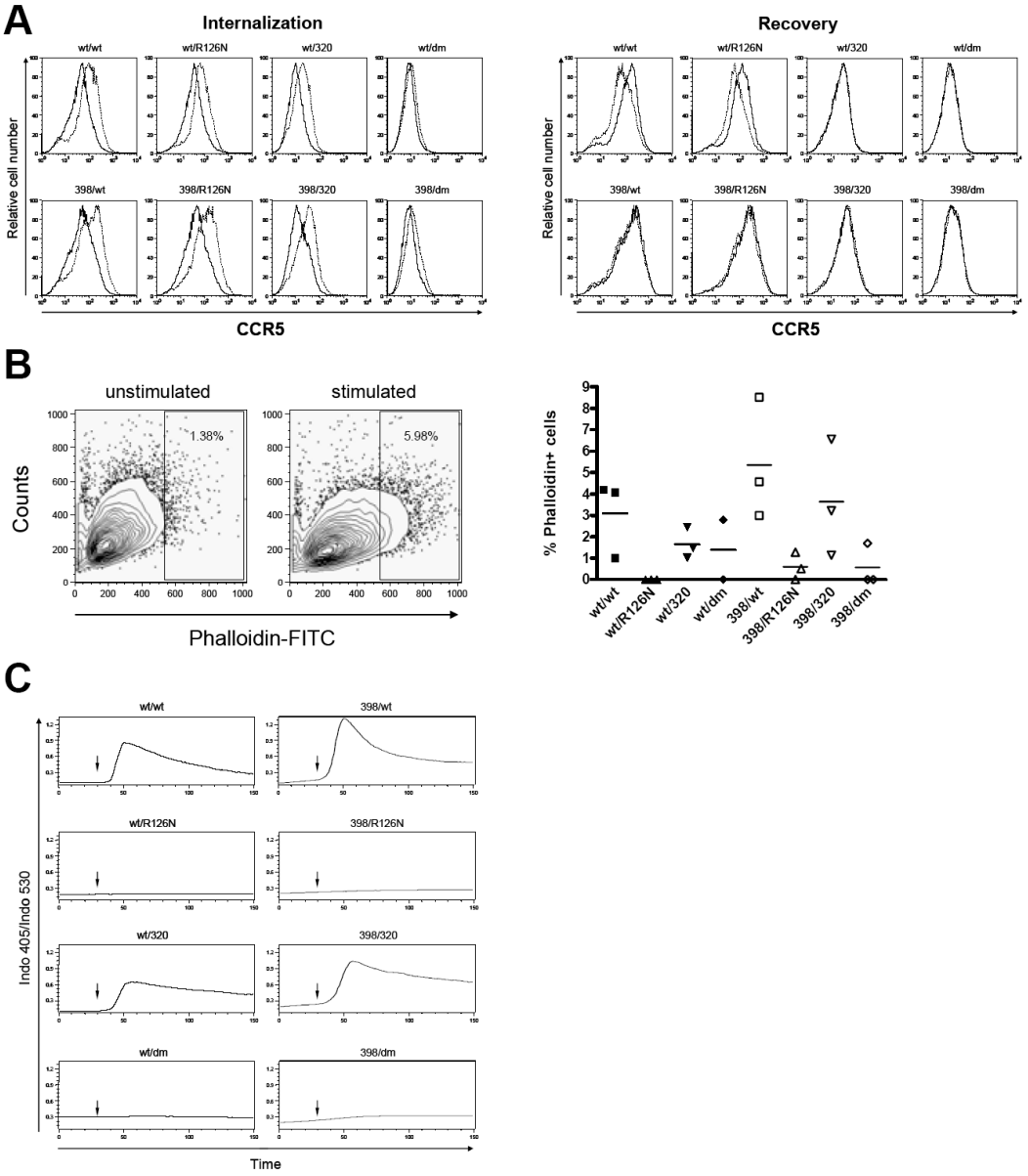

Fig. 5. Effect of CCR5 mutations on signalling. (A) Internalization and recovery of CCR5 in response to MIP1-β. Cells were stimulated with MIP-1β (1 μg/ml; solid lines) or not (dashed lines). After 2 h of stimulation, half of the cells were analyzed for CCR5 internalization by flow cytometry (left panel). The other half of the cells were thoroughly washed, incubated for another 4 h, and then analyzed for CCR5 recovery (right panel). Results are representative of four experiments. (B) CCR5-mediated actin polymerization upon MIP1-β stimulation. Actin polymerization was assessed after stimulation with MIP1-β (1 μg/ml) for 15 s by flow cytometric analysis of phalloidin-FITC binding. Left panel shows results for cells expressing CD4.398 and CCR5.wt for a representative experiment; unstimulated cells were used to determine background activity. Right panel shows results after subtraction of background activity of three experiments. (C) Intracellular calcium mobilization upon MIP1-β stimulation. Cells were loaded with Indo-1 AM and acquired on a LSR II flow cytometer. After obtaining a baseline for 30 s, MIP1-β (500 ng/ml) was injected (arrow), and cells were acquired for another 2 min. Data are presented as mean Indo-405/Indo-530 fluorescence emission versus time (in s). An increase in calcium is detected as an increase in Indo-1 fluorescence at 405 nm and as a decrease at 530 nm. Results are representative of two experiments. In (B) and (C), no response was observed in cells lacking CCR5 expression (data not shown).

CD4 signalling has an inhibitory effect on early reverse transcription.

To examine the effect of disrupting signalling through CD4 and/or CCR5 on the efficiency of early, post-entry events in the HIV infection process, we quantified early reverse transcripts 6 h p.i. by real-time PCR after inoculation of the cell lines with two R5 HIV molecular clones (49.5 [290] and YU.2 [291]) and three R5 HIV plasma-derived patient isolates [292].

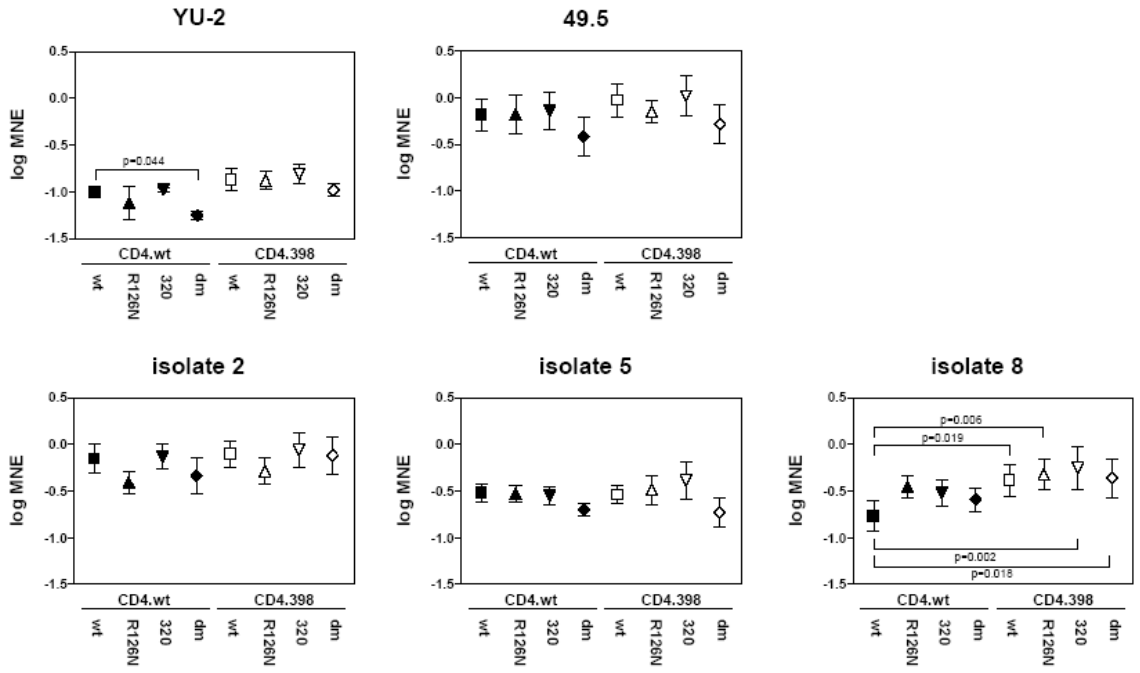

Fig. 6. Increased efficiency of early reverse transcription by disruption of CD4 signalling. Cells were inoculated with the R5 molecular clones YU-2 and 49.5 and three different R5 patient isolates. Quantities of early reverse transcripts were determined 6 h p.i. by real-time PCR. Expression of early reverse transcripts was normalized to that of the housekeeping gene beta-actin (MNE). Data show logarithmised MNE and represent mean ± SEM of (n = 4–6). Levels of early reverse transcripts in cognate and parental cells lacking either CCR5 or both CD4 and CCR5, respectively, were ≥ 10-fold lower than those in cells expressing both CD4 and CCR5 (data not shown). P-values are shown for significantly different levels of early reverse transcripts compared to those in the wt/wt cell line using Dunnett post-hoc test.

Two main observations were made: First, for all HIV strains tested but isolate 8, quantities of early reverse transcripts in wt/dm cells were lower than those in wt/wt cells; this difference was statistically significant for YU-2 (Fig. 6). A similar difference was observed between 398/dm and 398/wt cells, at least for YU-2, 49.5, and isolate 5, albeit non-significantly. This pattern was reminiscent of the considerably reduced cell surface expression of CCR5.dm relative to that of CCR5.wt (Fig. 2). Of note, low levels of receptor expression may limit HIV

entry into cells [293-300]. Thus, we quantified cell entry of YU-2 with a recently described enzymatic fluorescence-based assay [301]. Indeed, the extent of viral entry into wt/dm cells was considerably smaller than that into wt/wt cells (Fig. 7). Thus, the decreased quantities of early reverse transcripts in cells expressing CCR5.dm likely reflect a lower level of HIV cell entry due to a low number of CCR5 molecules and, therefore, receptor complexes available on the cell surface.

Second, and more importantly, levels of early reverse transcription in 398/wt cells (all viruses but isolate 5) and 398/320 cells (all viruses) were higher compared to that in wt/wt cells; for isolate 8, these differences reached statistical significance (Fig. 6). HIV entry into 398/wt and 398/320 cells is expected to be decreased or similar relative to that into wt/wt cells because basal cell surface expression of CD4.398 was lower than that of CD4.wt (Fig. 2), and CD4 cell surface expresion was not altered 6 h p.i. (data not shown). Furthermore, disruption of CD4/CCR5 signalling appears not to affect viral cell entry [147,149,276-279]. Thus, our data suggest that disruption of signalling through CD4 increases the efficiency of early reverse transcription.

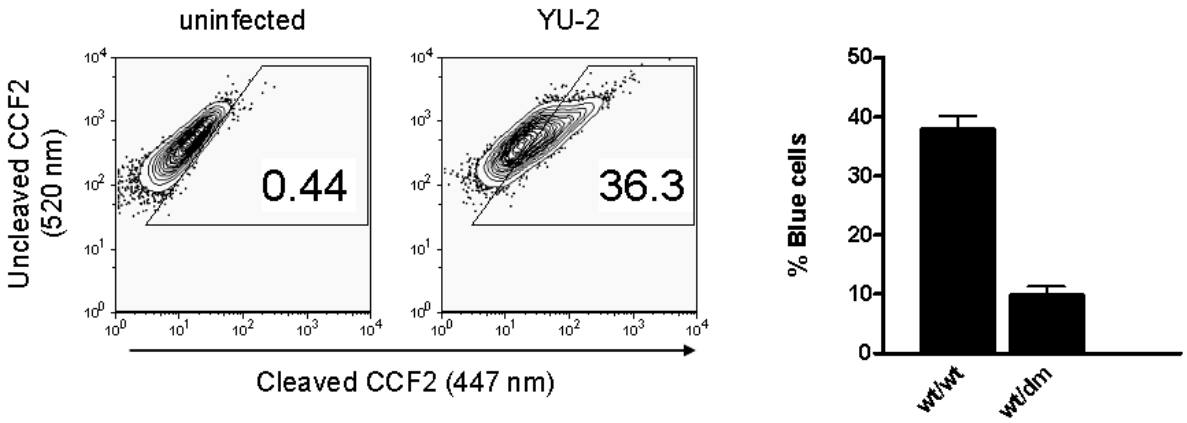

Fig. 7. Decreased entry of YU-2 into wt/dm cells. Cells were challenged or not with YU-2 virions containing BlaM-Vpr, loaded with CCF2/AM dye, and analyzed for viral cell entry by flow cytometry. Left panel shows representative results for CD4.wt/CCR5.wt cells; a gate was set based on the fluorescence of uninfected cells to determine background fluorescence. Right panel shows percentage of cells displaying increased blue fluorescence (447 nm) indicating viral cell entry after subtraction of background fluorescence. Data are given as mean ± SEM of two experiments.

Quantities of early reverse transcripts were higher in 398/320 cells than in 398/wt cells (this difference was also observed in cells co-expressing CD4.wt and seen with all strains used), suggesting that the effect of CD4 signalling is modulated by signalling through the C-terminal part of CCR5. However, there was no statistically significant difference (after Bonferroni correction) in the amount of early reverse transcripts between the cognate cell lines, indicating that disruption of signalling through CCR5 does not have a major effect on early reverse transcription.

Taken together, early HIV replication seems to be negatively regulated by CD4 signalling, but appears not to be substantially affected by CCR5 signalling.

CD4 and/or CCR5 signalling does not affect early–late steps of HIV replication.

To examine the effect of CD4 and/or CCR5 signalling on steps of the HIV replication cycle after early reverse transcription, we first determined the percentage of cells positive for the HIV core protein p24 by flow cytometry two days after inoculation with the molecular clones YU-2 and 49.5 and the patient isolates 2, 5 and 8. The percentage of $p24^+$ cells is a marker of productive infection and reflects the efficiency of several replication steps, including reverse transcription, integration, transcription and/or translation. In these experiments, cells were treated with Enfuvirtide, a fusion inhibitor, to ensure only one round of HIV replication.

For all HIV strains tested but 49.5, significant differences in the percentage of productively infected cells between one or several mutant cell lines and wt/wt cells were found: productive infection of 398/wt cells was significantly increased with YU-2, isolate 2, and isolate 8, and that of 398/320 cells was significantly higher with YU-2, isolates 2, 5, and 8 (Fig. 8). This pattern was reminiscent of the higher levels of early reverse transcripts in these cells compared to those in wt/wt cells (Fig. 6). Indeed, statistical analysis (unpaired t-test Bonferroni correction) revealed no difference between the patterns of early reverse transcription and productive infection. Thus, the significantly higher levels of productive infection of 398/wt and 398/320 cells relative to those of wt/wt cells likely reflect the greater amounts of early reverse transcripts in these cells. In other words, CD4 and/or CCR5 signalling does not seem to modulate late reverse transcription, integration, transcription and/or translation.

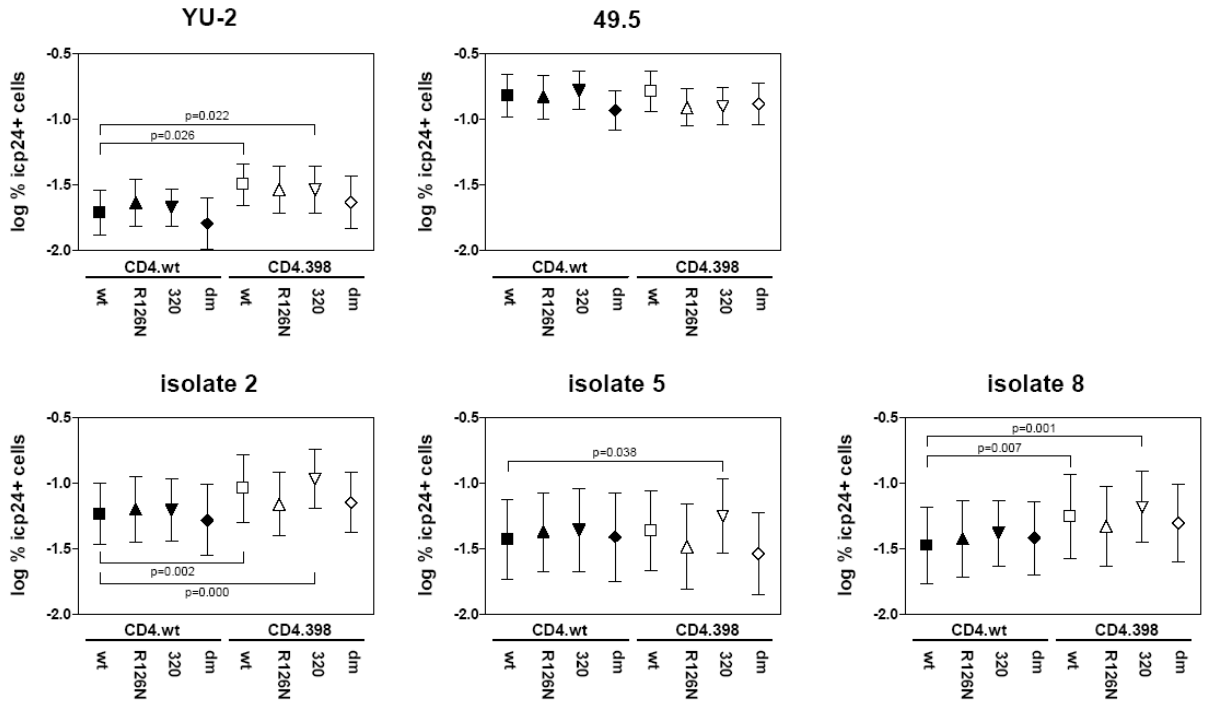

Fig. 8. Effect of CD4/CCR5 mutations on productive HIV infection. Cells were inoculated with the molecular clones YU-2 and 49.5 or patient isolates 2, 5, or 8 and analyzed for intracellular expression of the HIV p24 core protein on day 2 p.i. by flow cytometry. Background staining was determined with mock-infected cells and subtracted. Cognate and parental cells lacking either CCR5 or both CD4 and CCR5, respectively, were not productively infected (data not shown). After inoculation with virus, cells were treated with Enfuvirtide. Data show the logarithmised percentage of $p24^+$ cells (background subtracted) and represent mean ± SEM (n = 5–6). P-values are shown for significantly different levels of productively infected cells compared to those in the wt/wt cell line using Dunnett post-hoc test.

Second, we assessed the effect of the CD4/CCR5 mutations on the amount of virus produced by the infected cells by measuring the p24 concentration in the culture supernatant of cells on days 2 and 6 p.i. by ELISA. Cells were treated throughout the experiments with Enfuvirtide. For all five HIV strains, virus production in 398/wt and 398/320 cells was lower than that in wt/wt cells; a statistically significant difference was found for 49.5 between 398/320 cells and wt/wt cells (Fig. 9). This is directly opposite to the higher relative levels of early reverse transcription (Fig. 6) and productive infection (Fig. 8) in these cells. To explain this result, it has to be considered that the presence of CD4 on the cell surface poses several problems for post-entry steps of the HIV replication cycle, including retention of budding virions [302]. Importantly, in contrast to CD4.wt, CD4.398 is not down-modulated upon stimulation with

PMA (Fig. 4) or several days after infection with HIV (data not shown). Thus, the lower amount of virus produced in cells expressing CD4.398 is likely explainable by an impaired release of virions due to the permanent presence of this CD4 variant rather than by the disruption of signalling through CD4.

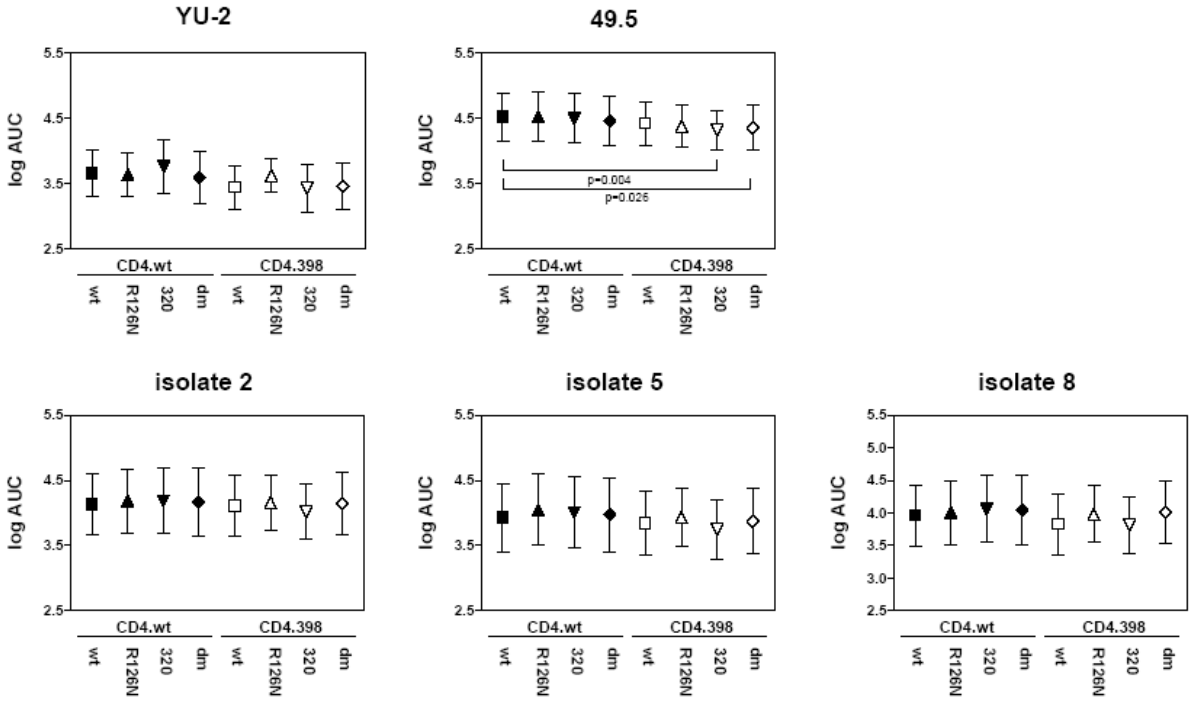

Fig. 9. Reduced virus production in cells expressing CD4.398. Cells were infected and treated as described in the legend to Fig. 8, and p24 concentrations in the cell culture supernatants were analysed by ELISA on day 2 and 6 p.i.. Data show the logarithmised area under the curve (AUC) and represent mean ± SEM (n = 5–6). P24 concentrations in cell culture supernatants from HIV-inoculated cells expressing only CD4 but not CCR5 HIV were negligible (data not shown). P-values are shown for significantly different levels of virus production compared to those in the wt/wt cell line using Dunnett post-hoc test.

4.3 Discussion

Each of the components of the HIV receptor complex is involved in signalling, raising the question of whether HIV exploits these signalling pathways to facilitate its own replication. In this work, we established an experimental system consisting of a panel of cell lines stably expressing different combinations of wt and mutant CD4 and CCR5, which were infected with two R5 HIV molecular clones and three R5 HIV plasma-derived patient isolates. The influence of signalling through the receptor complex on HIV replication was assessed by comparing the efficiency of different steps of the HIV life cycle in the mutant cell lines relative to that in the CD4.wt/CCR5.wt cell line. Our data suggest that early reverse transcription is negatively regulated by signalling through CD4, but not substantially modulated by CCR5 signalling, and that later steps of the HIV replication cycle are not directly affected by CD4/CCR5 signalling.

Our experimental system was designed to take into account that CD4 and CCR5 are physically associated [282], and that there is a functional cross-talk between the receptors: the activation of CD4 by IL-16 results in enhanced signalling through CCR5 [285]; conversely, CCR5 increases a migratory response induced by IL-16 through CD4 in Th1 cells [283]; furthermore, a reciprocal cross-desensitization between the two receptors has been reported [284].

We first characterized our cell lines phenotypically and functionally. Cell surface expression of CD4.398 was lower than that of CD4.wt. As reported, CCR5.R126N and CCR5.wt were expressed similarly [277,280,281]. The lower levels of CCR5.320 expression seen by us and others [276,303] are likely explained by disruption of two motifs within the C-terminal tail of CCR5, which are critical for its cell surface expression (i.e., a membrane-proximal basic domain at residues 314–322 [303] and a cysteine cluster (Cys-321, -323, and -324) [279,303]. Cell surface expression of an as yet uncharacterized CCR5 variant, CCR5.dm (i.e., R126N in the DRY motif and a deletion after residue 320), was even lower than that of CCR.320. The DRY motif and the C-terminal cysteine cluster constitute a bipartite motif that is required for binding of β-arrestins [304]. Thus, β-arrestins may also be important for optimal cell surface expression of CCR5, independent of their role in agonist-promoted internalization and trafficking of 7TM G protein-coupled receptors [305]. We also generated cell lines co-expressing CD4 and a truncated CCR5 variant which was made by introducing a premature stop codon after Leu-308. However, this CCR5 variant was barely expressed at the cell surface (data not shown) and was not investigated further.

Wt and mutant CD4 were signalling-intact and -defective, respectively: stimulation with PMA resulted in down-modulation of CD4.wt, but not CD4.398, as reported [147]; likewise, CD4.wt, but not CD4.398 was down-modulated upon infection with HIV (data not shown). In line with previous reports [288,289], CCR5.wt, CCR5.R126N, and CCR5.320 showed efficient internalization upon incubation with MIP-1β and recovery upon chemokine withdrawal. In contrast, the weak endocytosis of CCR5.dm is most likely explained by its inability to bind β-arrestins. CCR5.wt and CCR5.320 consistently mediated actin polymerization when stimulated with MIP1-β, whereas CCR5.R126N-mediated polymerization was negligible, and CCR5.dm mediated a variable response. Actin cytoskeletal rearrangements are induced upon coupling of 7TM receptors to G proteins, which depends on the DRY motif. Similarly, CCR5.wt and CCR5.320 mediated effective chemotaxis upon MIP-1β stimulation [280,288,289], whereas CCR5.R126N and CCR5.dm did not (data not shown). In accord with published reports [276,277,280,281,288], CCR5.R126N and CCR5.dm failed to mobilize calcium; CCR5.320 displayed a smaller but longer response than wt CCR5. In all three assays (i.e., actin polymerization, calcium mobilization, and chemotaxis), responses mediated by CCR5.wt were more pronounced in cells co-expressing CD4.398 than in those with CD4.wt. This finding is compatible with data showing that CD4 induces a Lck-dependent signal that results in cross-desensitization of CCR5 [284]. Thus, phenotypic and functional characterization of the cell lines indicated CD4/CCR5 cross-talk and validated our experimental system to investigate the role of CD4/CCR5-mediated signalling on HIV.

We analysed several parameters of HIV replication in our cell lines: 1) the amount of early reverse transcripts by real-time PCR as a marker of early HIV replication; 2) the percentage of $p24^+$ cells by immunostaining/flow cytometry two days p.i. as a marker for productive infection, reflecting the efficiency of reverse transcription, integration, transcription, and translation; and 3) the concentration of p24 in the cell culture supernatants over time by ELISA as a marker for virus production, reflecting the efficiency of all steps of the replication cycle, including virus assembly and budding. Furthermore, unlike most of the previous studies examining the effect of signalling on HIV replication, we used several HIV strains, i.e., two R5 molecular clones and three R5 plasma-derived patient isolates, in our infection experiments.

Analysis of early reverse transcription revealed two different patterns: First, quantities of early reverse transcripts were lower in cell lines expressing CCR5.dm than in cell lines expressing CCR5.wt. Importantly, CCR5.dm had a strongly reduced cell surface expression relative to that of CCR5.wt and low levels of receptor expression are a limiting factor for HIV

entry into cells [293-300]. Indeed, analysis of YU-2 cell entry showed considerably lower levels in wt/dm than in wt/wt cells. Therefore, the low level of early reverse transcripts in cells expressing CCR5.dm is likely explainable by a low level of HIV cell entry due to the low cell surface expression of this CCR5 variant rather than by disruption of signalling through CCR5.

Second, levels of early reverse transcription were generally higher in cells expressing CD4.398 than in cells with CD4.wt. This difference was most pronounced for isolate 8. Unfortunately, the enzymatic fluorescence-based assay used to measure cell entry of YU-2 is not readily applicable to primary HIV isolates since the production of viral particles containing BlaM-Vpr involves proviral DNA, which is available for several molecular clones, but generally not for primary isolates. Furthermore, for unknown reasons, uninfected cells expressing CD4.398 displayed a high background fluorescence when loaded with CCF2/AM dye, preventing us from accurately quantifying HIV entry into these cells. Thus the significantly higher levels of early reverse transcription in cells expressing CD4.398 could in fact reflect a higher level of viral cell entry. However, basal cell surface expression of CD4.398 was lower compared to that of CD4.wt, and this expression pattern was not altered 6 h p.i., i.e., at the time point when cells were analysed for early reverse transcription (data not shown). Moreover, disruption of CD4/CCR5 signalling seems not to affect viral cell entry [147,149,276-279]. Therefore, HIV entry into cells expressing CD4.398 is expected to be rather decreased as compared to cells expressing CD4.wt. Thus, disruption of signalling through CD4 appears to increase the efficiency of early reverse transcription, suggesting that signalling through CD4 inhibits HIV replication at this early, post-entry step.

To assess whether the negative effect of CD4 signalling on early HIV replication was modulated by signalling through CCR5, we compared the levels of early reverse transcripts in 398/R126N, 398/320, and 398/dm cells to those in 398/wt cells. There was a tendency towards higher levels in 398/320 cells than in 398/wt cells. This suggests that signalling through the C-terminal part of CCR5 may moderately modulate the effect of CD4 signalling.

Flow cytometric analysis of the percentage of $p24^+$ cells on day 2 p.i. revealed a higher level of productive infection of cells expressing CD4.398 as compared to cells expressing CD4.wt. This pattern was similar to that of early reverse transcription, and indeed, statistical analysis showed no difference betweeen these two parameters. Therefore, the higher level of productive infection likely reflects the greater amount of early reverse transcripts in these cells. In other words, CD4 signalling does not seem to directly affect late reverse transcription, integration, transcription, and translation.

Finally, measurement of p24 in the cell cultures over time revealed a lower level of virus production by 398/wt and 398/320 cells relative to that by wt/wt cells (significant for 49.5). This pattern was directly opposite to that of early reverse transcription and productive infection, the levels of which were higher in these cells. A possible explanation for this phenomenon is an increased retention of budding virions in these cells. CD4 is normally down-regulated by HIV to ensure efficient replication, since its presence prevents among others efficient release of viral particles [302]. However, down-modulation of CD4.398 upon stimulation with PMA or several days after infection with HIV (data not shown) is impaired.

The role of signalling through CD4 or CCR5 for HIV replication has been a matter of debate. Several investigators reported enhanced HIV replication by CD4 signalling [147-151], whereas one study demonstrated a negative effect [146]. Likewise, CCR5-mediated signalling had a positive effect on HIV replication in one study [281], but not in another [280]. Our data are partly in line with the study demonstrating a negative effect of CD4 signalling on HIV replication [146]. However, in this study, viral replication was modulated on the level of productive infection and virus production, whereas viral entry and reverse transcription were not altered. In contrast, we observed modulation of early reverse transcription by CD4 signalling, but no direct effect on steps thereafter. As for CCR5, our data suggest a minor, if any role of signalling through this receptor for early reverse transcription.

In conclusion, our data suggest that CD4 signalling has an inhibitory effect on early HIV replication, whereas CCR5 signalling does not play a major role for this modulation, and that later steps of the HIV replication cycle are not directly affected by CD4/CCR5 signalling.

4.2 Materials and methods

DNA constructs. pIRESpuro-CD4.wt and pIRESpuro-CD4.398 were generated by cloning the sequences encoding CD4.wt and CD4.398, respectively, into pIRESpuro (Clontech Laboratories). CD4.398 was obtained as described [306] by site-directed mutagenesis (Quick Change Site-Directed Mutagenesis kit, Stratagene). For constructs encoding CCR5 variants, CCR5 sequences were cloned into the retroviral vector pEneo (from B.C. Schaefer [307]). CCR5.320 was made by introducing a premature stop codon after Phe-320 in CCR5.wt. CCR5.R126N and CCR5.dm were generated by changing Arg-126 to Asn in CCR5.wt and CCR5.320, respectively, by site-directed mutagenesis. All plasmids were confirmed by sequencing.

A2.01-CD4/CCR5 cell lines. A2.01 cells (NIH AIDS Research and Reference Reagent Program) were genetically complemented with CD4 by electroporation with a Bio-Rad Gene Pulser (Bio-Rad) and subsequently with wt or mutated CCR5 by lentiviral transduction through spinoculation at 1,200 × g for 2 h at 25°C. Cells were grown in culture medium (RPMI1640, 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, via Invitrogen AG), 0.5 μg/ml puromycin (Sigma), and 800 μg/ml neomycin (Gibco).

Virus production. Lentiviruses encoding wt or mutant CCR5 were produced by transfecting 293T cells with pMoMLV, pMD2.G (from D. Trono, University of Geneva), and the respective pEneo-CCR5 construct. For virions containing BlaM-Vpr, 293T cells were transfected with pYU-2, pCMV-BlaM-Vpr (from M. Cavrois, [301]), and pAdVAntage (Promega). Culture supernatants were concentrated by Amicon Ultra centrifugal filters (Millipore). 49.5 and YU-2 stocks were produced with p49.5 [290] and pYU-2 [291], respectively. p24-Gag levels of virus stocks were determined as described [308]. Plasmids were obtained through the NIH AIDS Research and Reference Reagent Program unless otherwise noted. 293T cells were transfected by the calcium phosphate method (Promega, via Catalys AG). Patient isolates (from A. Trkola, [292]) were expanded on PBMC and titrated on A2.01 cells expressing CD4.wt and CCR5.wt. The 50% tissue culture infectious dose of the virus stocks was determined -as described [292].

Receptor expression. Cells were stained with a monoclonal (mAb) against CD4 (clone RPA-T4), CCR5 (2D7), or isotype immunoglobulins (BD Biosciences-Pharmingen) and analyzed by flow cytometry.

Cell proliferation. Cells were stained with 0.05 μM carboxy fluoroscein succinimidyl ester (CFSE) (Molecular Probes, via Invitrogen) and analyzed 1 and 4 days later by flow cytometry immediately after staining.

CD4 down-modulation. Cells were starved in selective medium with 0.1% FCS for 24 h, incubated in culture medium with or without 0.05 μg/ml phorbol myristic acetate (PMA) (Sigma) for 4 h at 37°C in humidified air with 5% CO_2, and analyzed for CD4 cell surface expression.

CCR5 internalization. Cells starved for 24 h were analyzed for CCR5 cell surface expression after stimulation with 1 μg/ml MIP1-β (PeproTech EC) for 2 or 4 h after chemokine withdrawal.

Actin polymerization. Cells starved for 24 h and prewarmed for 10 min at 37°C were stimulated in 400 μl of RPMI 1640 with 1 μg/ml MIP1-β for 15 s at 37°C. The reaction was stopped with 100 μl of a perm/stain/fix solution (PBS, 0.1% saponin, 0.5 μg/ml phalloidin-FITC (Sigma), and 20% paraformaldehyde). Cells were incubated on ice for 10 min, washed, and analyzed by flow cytometry.

Flow cytometry. Cells were acquired on a FACSCalibur (BD Biosciences), unless otherwise stated and data analyzed with FlowJo software (Tree Star).

Calcium mobilization. Cells were resuspended in HBSS (Cambrex Bioscience, via BioConcept) with 1 mM Ca^{+2}, 1 mM Mg^{+2}, 4 mM Probenicid (Sigma), and 1% FCS at 1×10^6 cells/ml, loaded with 2 μg/ml Indo-1 penta-acetoxymethyl ester (Indo-1 AM; Molecular Probes), and incubated at 37°C for 30 min. Cells were acquired on a LSR II (BD Biosciences; violet bandpass filter centered at 395 nm and blue bandpass filter centered at 500 nm) at 200–300 cells/s for 30 s, MIP1-β (500 ng/ml) was injected, and acquisition continued for another 2 min.

Viral cell entry. HIV cell entry was examined with an enzymatic fluorescence-based method essentially as described [301]. Briefly, 2×10^6 cells were incubated with virions containing BlaM-Vpr (400−500 ng p24-Gag) at 37°C for 2 h, washed once in CO_2-independent medium (Gibco), and then loaded with CCF2/AM dye (Panvera, via Invitrogen) for 1 h at room

temperature (r.t.). After one wash with CO_2-independent medium, the BlaM reaction was allowed to develop for 16 h at r.t. Cells were then washed once in PBS and fixed in 1.2% paraformaldehyde for 24 h at 4°C. Finally, cells were washed twice and resuspended in PBS, and analyzed on a FACS Aria (BD Biosciences).

Quantitation of early reverse transcription. Quantitative, real-time PCR was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). The R-U5 region, representative of minus-strand strong-stop vDNA, was detected using primers HIV-SS-F4, HIV-SS-R4 (1 μM each), probe P-HUS-SS1 (100 nM) [309] and qPCR Master mix (Eurogentec). A2.01 cells were infected with HIV isolates as described below. Total DNA was isolated with QIAmp DNA blood Mini kit as per the manufacturer's recommendations (Qiagen). In each PCR reaction, 200 ng of total DNA was amplified in a final volume of 15 μl: 50°C for 2 min, 95°C for 10 min, followed by 60 cycles of two-step amplification regimens (95°C for 15 s and 60°C for 1 min). The beta-actin housekeeping gene was amplified in separate reactions with a purchased ready-to-use primer/probe kit (Eurogentec). All reactions were performed in duplicate. Specific amplification efficiencies were determined by measuring serial dilutions and included in the data analysis.
Data from real-time quantitative PCR were analyzed in two steps. First, the mean normalized gene expression (MNE) (i.e., normalized to the housekeeping gene) for every sample was determined using the software application Q-Gene (calculation procedure for MNE 2) [310].

HIV infections. Cells (2×10^5/well in 96-well U-bottom plates) were spinoculated in duplicate with virus (0.5 (49.5) or 5.0 ng (YU-2) of p24-Gag; patient viral isolates were used at an MOI of 0.001) at 1,200 x g for 2 h at 25°C, cultured for 4 h, and extensively washed. For quantitation of early reverse transcripts (Fig. 6), DNA was extracted from the cells 6 h post-infection (p.i.) and analysed by real-time PCR as described above. For analysis of productive infection (Fig. 8), cells were cultured for 2 days, permeabilized/fixed with Cytofix/Cytoperm (BD Bioscience), and stained for intracellular p24 with a p24-specific mAb (KC57, Beckman Coulter). To control for background staining, the percentage of $p24^+$ cells in mock-infected cells was substracted from that in HIV-infected cells. Virus production (Fig. 9) was analysed by measuring the p24 concentrations in the cell-culture supernatants on days 2 and 6 p.i. by ELISA [308]. In all experiments, cells were treated with Enfuvirtide (Fuzeon, 50 μg/ml; Roche) after inoculation with virus; Enfuvirtide was maintained thereafter in the cell cultures

throughout the experiments. Cognate and parental cell lines (i.e., lacking either CCR5 or both CD4 and CCR5) were used as controls in all infection experiments.

Statistics. Values of early reverse transcription (MNE), productive infection (percentage of $p24^+$ cells), and virus production (area under the curve (AUC) of p24 concentrations in the cell culture supernatants analysed by ELISA on day 2 and 6 p.i.) were analyzed separately for each virus (molecular clones YU-2, 49.5, patient isolates 2, 5, 8). Mixed two-way analysis of variance with random factor experiment and fixed factor cell line was applied to logarithmized data. Post-hoc comparisons were performed using Dunnett- and Bonferroni-tests where appropiate. Differences between cell lines were compared between early reverse transcription and infection using unpaired t-test with Bonferroni correction. The program SPSS 13 (SPSS Inc. Chicago, IL) was used for all analyses. P-values $p < 0.05$ were considered statistically significant.

4.4 Acknowledgements

We thank the Institute of Medical Microbiology, University of Zurich, for kindly providing the Gene Pulser for electroporation of A2.01 cells. We also thank the Institute of Pathology, University of Bern, in particular C. Vallan, for kindly providing the LSR II and for assistance in measuring calcium flux. We are also grateful to E. Niederer (ETH Zurich) for help with analyzing viral entry on a FACS Aria. We acknowledge B.C. Schaefer for kindly providing us with the retroviral vector pEneo and D. Trono for contributing pMD2.G. M. Cavrois is acknowledged for kindly providing pCMV-BlaM-Vpr and technical advice for quantifying viral entry. We thank P. Rusert and A. Trkola for donating patient isolates. We gratefully acknowledge the contribution of several reagents by the NIH AIDS Research and Reference Reagent Program. This work was supported by grants from the Kurt and Senta Herrmann Foundation, OPO Foundation, and Olga Mayenfish Foundation.

5

Conclusions and Outlook

5.1 Current HIV mouse models

HIV is responsible for the progressive immunodeficiency which culminates in AIDS and which has resulted in an ongoing pandemic. The available anti-retroviral drugs efficiently suppress HIV but reveal major limitations such as side effects, emergence of resistant strains, psychic distress and high costs.

Since the start of the HIV pandemics, HIV research has been hampered by the lack of a small animal model. A small versatile HIV animal model would be of utmost value for examining HIV pathogenesis and testing novel treatment approaches. Notably, HIV is a human specific pathogen and does not infect and replicate in rodents such as mice or rats. To overcome this limitation mouse models such as hu-PBL-SCID [68,174] and SCID-hu mice [56,175] have been developed in which human hematopoietic cells and tissues are transplanted into mice that are compromised in their capacity to reject xenogenic grafts. Both models sustain HIV infection and replication *in vivo*. However, in hu-PBL-SCID mice xeno-reactivity and successive loss of human leukocytes limit infection to a relatively short time frame, and infection is skewed towards R5- tropic HIV strains [72,176]. In SCID-hu mice HIV infection can be observed for extended times; however, availability of transplantable human fetal organs is restricted for practical and ethical reasons, and HIV pathology in these mice is mainly limited to the tissue implants, with naïve T cells being preferentially susceptible to CXCR4-tropic strain infection [9,59,177]. One of the major improvements was achieved by using NOD/SCID *Il2rg*$^{-/-}$ (NOG) and newborn Rag2$^{-/-}$ *Il2rg*$^{-/-}$ (RAG-hu) mice as recipients for human hematopoietic stem and progenitor cell grafts because this approach allows tremendously high engraftment levels and efficient intra-thymic *de novo* development of human T cells [49,51,54,178], which are the targets for HIV.

We established the humanized Rag2$^{-/-}$ *Il2rg*$^{-/-}$ mice based on the transplantation of human CD34^{+} at newborn age (RAG-hu mice [49]) as a new tool to study HIV infection and pathogenesis *in vivo*. RAG-hu mice have several significant advantages over other available mouse models: they sustain *de novo* T cell generation from CD34^{+} cells with a broad repertoire over at least 6 months, T cells are not activated by xenogeneic tissue and populate secondary lymphoid organs as lymph nodes and spleen where they encounter newly formed human B cells and antigen presenting cells, human cells in concert built up secondary lymphoid organ structures, and some human primary immune responses were detectable[49,178]. Finally, compared to SCID-hu mice where multiple fetal organs are transplanted, RAG-hu

mice represent a very straight-forward, easily applicable and ethically less problematic model. We show that RAG-hu mice are indeed an ideal model supporting long-term, high-titer, and lymphoid organ disseminated HIV replication closely resembling HIV infection in humans, thus allowing to study HIV pathogenesis with respect to effects on the thymic function, bone marrow and secondary lymphoid organs [82] **(chapter2)**.

Shortly after we have pubished our work, there were a number of reports which have corroborated our data showing that hNOG, RAG-hu, and RAG-hu mice co-transplanted with human thymus/liver are valuable as models for HIV infection [82,203-206] (Table 2).

The comparison of all these humanized mouse models indicate that 1) hNOG mice seem to allow higher and more stable levels of chimerism and, thus, better immune responses than RAG-hu mice but long-term studies are limited in hNOG mice. Notably, in hNOG, HIV infection could only be monitored for 43 days due to wasting and due to their short lifespan [204]. The same group thereafter reported that myeloablation procedures were not required for human cell engraftment and that hNOG mice without myeloablation stably survived beyond 300 days after transplantation permitting long-term studies on HIV pathogenesis [311]. However, a direct comparison between RAG-hu and hNOG mice is lacking; that 2) the bone marrow/liver/thymus (BLT) model is the only model that currently allows to reproducibly study intrarectal transmission or gastrointestinal tract associated infection [206]. The BLT model is a combination of the SCID-hu and the human $CD34^+$ cell-transplanted NOD/SCID model: the bone marrow is reconstituted with HSC, which generate e.g., T-cell progenitors that repopulate the implanted human thy/liv organoid [202]. The BLT model, incorporates the best aspect of each model and develops a significantly more complete human immune system including reconstitution of the gut associated lymphoid tissue [202]. However, this model is extremely complex, labor intensive and costly; that 3) many different mouse strains, reconstitution procedures, infection protocols and combinations thereof are currently used. The optimal host strain, age and route of infection are not yet known. A thorough direct comparative analysis of the various model systems would need to be done.

Senior author	**Yamamoto**	**Garcia**	**Speck**	**Su**	**Akkina**	**Poluektova**	**Uittenbogaart**
Reference	204,311	202,206	82	205	203	312	313
Mouse strain	NOD/Shi-*scidIl2rg*$^{-/-}$	NOD/LtSz-*scid*	Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$	Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$	Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$	Balb/c *Rag2*$^{-/-}$*Il2rg*$^{-/-}$	H2^{d} *Rag2*$^{-/-}$*Il2rg*$^{-/-}$
Abbrevation	hNOG mice	BLT	RAG-hu	RAG-hu	RAG-hu	HIS	HIS
Reconstitution	i.v. injection of cord blood (CB) derived CD34^{+} cells into 6-10 weeks old mice	i.v. injection of fetal liver (FL) derived CD34^{+} cells into SCID-hu mice	intrahepatic (i.h.) injection of CB derived CD34^{+} cells into newborn mice	i.h. injection of CB or FL derived CD34^{+} cells into newborn mice	i.h. injection of FL derived CD34^{+} cells into newborn mice	i.h. injection of CB derived CD34^{+} cells into newborn mice after busulfam administration	i.p. injection of FL derived CD34^{+} cells into newborn mice
HIV-1 strain Infection protocol	JRCSF, SHIV-C2/1; i.v. approx. 5x10^{4} $TCID_{50}$	HIV-1_{LAI} ; intrarectally approx. 1x10^{5} $TCID_{50}$	YU-2, NL4-3; i.p. approx. 2x10^{6} $TCID_{50}$	JRCSF, NL4-3; NL4-R3A, i.v. approx. 2x10^{4} $TCID_{50}$	Ba-L, NL4-3, i.p. approx. 1x10^{5} $TCID_{50}$	NL4-3, ADA; i.p. approx. 1x10^{4} $TCID_{50}$	NFN-SX(SL9); i.p. approx. 1x10^{3} $TCID_{50}$
advantage	high and stable chimerism stable infections some immune responses to HIV	reconstitution of gut intrarectal transmission human innate immunity	long-term infection				
limitations	short life-span (7 months), wasting only 40 days of infection followed	complex, labor intensive	weak immune responses			no immune responses	no immune responses

Table 2. Comparison of current HIV mouse models that are based on HSC transplantation.

5.1.2 Expected value of these models.

The hNOG and RAG-hu models have distinct attributes which make them highly valuable for testing novel compounds and pharmacologic approaches aiming to prevent or treat HIV infection. Attributes are: high-titer viremia over months, easy monitoring of HIV RNA copy numbers in the blood and susceptibility to HIV strains with distinct co-receptor selectivity. This should allow to investigate the emergence of resistant strains upon long-term treatment and to investigate the effect of anti-retroviral treatment on established HIV infection. Notably, in rather all previous publications using the SCID mouse model, investigational drugs have been either applied prior or within days after HIV exposure which represents rather "pre-" or "post-exposure prophylaxis". We also believe that the human lymphoid like system permits to study immunomodulation (e.g., with cytokines) for enhancing the body's own immune defence.

Many laboratories including ours are aiming to establish the above mentioned models to study mucosal HIV transmission: vaginal and rectal transmissions are the primary mode of transmission worldwide, and blocking mucosal transmission would be a very critical step to halt the HIV pandemic. A small HIV mouse model which permits to study mucosal transmission is anxiously awaited from the HIV research community, especially for testing compounds (i.e., microbicides) that, when applied vaginally or rectally, will prevent HIV transmission. Our preliminary data on vaginal **(chapter 2)** and the data from Sun et al.[206] on intrarectal transmission are promising.

5.1.3 Future directions

The genetic manipulation of fetal $CD34^+$ cells prior to transplantation may open a new avenue to study the effects of individual gene products on HIV *in vivo* and therefore host-HIV pathogen interactions. In **chapter 2** we propose to investigate the *in vivo* significance of APOBEC cytidine deaminases, which pose a powerful mechanism of innate immunity. These models may also be used to investigate the role of chronic immune activation in disease progression which could extend our findings from **chapter 3.** There, we propose that sustained TLR7 stimulation by HIV ssRNA may directly contribute to immune activation, which is considered a major cause for progressive immunodeficiency in HIV infection. RAG-

hu mice may help to test the ability of specific TLR antagonists to reduce immune activation and thereby disease progression in HIV-infected mice.

One of the major limitations is the lack of human MHC expression in non-hematopoietic tissue and in the mouse thymus, which is required to support the selection and survival of T cells following human stem-cell engraftment [41]. Human thymocytes in humanized mice possibly are positively selected on mouse and negatively selected on both mouse and human MHC **(chapter 2)**. Human T cells positively selected on mouse MHC (H2) will not be able to recognize antigens presented by HLA-expressing human antigen-presenting cells in the periphery [39]. Moreover, emigrating T cells depend on homeostatic factors such as cytokines, which may not provide the adequate stimuli because of defects in cross-species interactions. These limitations hinder the generation of robust human T-cell responses and adequate T-cell help, which in turn hinders the generation of human B-cell responses against T-cell dependent antigens such as HIV. Nevertheless, the observation that B-cell maturation to IgG producing cells only occurred in mice with T-cell development [42] and the detection of specific, although inconsistent and low, B-cell responses [51,82], suggest some T-cell help at least. The lack of human follicular dendritic cells (FDC) may further hamper B-cell responses [49].

The expression of transgenic human MHC, the addition of human stroma cells with FDC differentiation capacity as well as the addition of human cytokines, growth factors, and chemokines might therefore improve the models value for vaccine testing. HLA-transgenic mice are currently being generated in the NOG strain [39]. Finally, *in vitro* expansion of haematopoietic precursor cells prior to transplantation may help to increase the cohort-size and to decrease the engraftment variability. This is important, because less than 10 animals can usually be transplanted from one graft, and inter- and intra-litter engraftment variability is high, probably due to donor-cell variability, pre-transplant cell handling, and the transplantation procedure itself. If these problems can be solved the possible use of these models will be enormous, and will impact on both basic and preclinical research, although it may rather reduce than replace the number of studies that are required in large animals and humans. Notably, humanized mice might be instrumental in vaccine and drug development against human pathogens such as HIV, Epstein-barr-, Dengue- or influenza virus, as they not only develop all types of human cells that are needed for viral infection and growth but also the major types of human cells that orchestrate adaptive immune responses [41].

5.2 The role of TLR signalling in immune activation

Chronic immune activation is a major cause for progressive immunodeficiency in HIV infection. The precise mechanism and the underlying trigger by which HIV causes immune activation remain poorly understood. There is growing notion that TLR activation may not only enhance antiviral immunity by activating the immune system (reviewed in [220]), but also play a pivotal role in aberrant immune activation seen in HIV infection [110]. In natural HIV infection, the immune system fails to eliminate the virus resulting in persistent ssRNA production, which can constantly induce TLR7/8 signalling. Thus, we hypothesized that TLR7/8 signalling is a main driver for chronic immune activation and thereby contributes to the progressive immunodeficiency observed in HIV infection **(chapter 3)**. To explore the effects of sustained TLR7 triggering on the molecular and cellular components of the lymphoid system of mice, we used the synthetic compound R848 which triggers TLR7/8 similarly to HIV ssRNA [114,118,120]. In conclusion, our data demonstrated that sustained TLR7 triggering in mice results in immune activation and disruption of the lymphoid system reminiscent of HIV associated pathology; Sustained triggering of TLR7 led to i) altered white blood cell counts, including profound lymphopenia and increased neutrophil and monocyte levels ii) immune activation and attenuated humoral immune responses, iii) disruption of the lymphoid structure, including splenomegaly, enlarged T and B cell zones, reduced marginal zone B-lymphocytes and a relative contraction of lymphoid subsets, iv) cytokine deregulation, v) thymic hypocellularity. We also showed that the observed effects were TLR7- and dose dependent, and that IRF-7 and IFNAR mediated signalling only partly contributed to R848 mediated pathology.

Our findings underline that HIV ssRNA itself probably contributes to persistent immune activation and pathology by signalling through TLR7, and explains in part the chronic immune dysfunction in HIV infection. Manipulating TLR7 triggering or down-stream signalling may be therapeutically valuable to reduce chronic hyper-immune activation and immune dysfunction. Proof of concept studies are now needed to test, whether blocking of TLR7/8 signalling can block immune activation and dysfunction in SIV-infected macaques and in HIV-infected humanized mice.

5.2.1 Immune-based therapies to decrease T-cell activation

Rizzardi et al. recently suggested that directly interfering with immune activation might positively affect immunologic outcomes in HIV acute infection. Treatment of patients with primary HIV infection with the immunosuppressive drug cyclosporine A coupled with antiretroviral therapy resulted in significantly greater $CD4^+$ T-cell recovery through at least 64 weeks of follow-up [314]. These findings further corroborate a causal role of T-cell activation in HIV pathogenesis. Nevertheless, treatment of patients with chronic HIV infection with immunosuppressive drugs either showed no or negative clinical benefit [315,316] probably due to side effects and unspecific and generalized suppressive effect on protective, antigen-specific immune responses. The ideal therapy would preserve protective immune responses while preventing generalized immune activation. Elucidation of the mechanisms by which HIV causes immune activation will help to develop such targeted immune-based therapies [103].

5.2.2 Immune activation and T-cell depletion

Several studies, including ours (chapter 3), identified potential underlying triggers of immune activation. Little is known, however, about how immune activation eventually causes T-cell depletion and progressive immunodeficiency.

For many years $CD4^+$ T-cell loss has been explained by the "tap and drain" hypothesis [79] in which $CD4^+$ cells are constantly eliminated by HIV (drain) and constantly replaced by homeostatic compensation (tap). A small imbalance between death and replenishment results in a gradual decrease of $CD4^+$ T cells over time. The "runaway hypothesis" is a candidate mechanism explaining this imbalance; immune activation and homeostatic proliferation drive infection, higher viral loads, more recruitment of cells into an activated state, further infection events, and slow runaway depletion of $CD4^+$ T cells [317]. Yates et al, however, rejected this runaway hypothesis, because their mathematical model predicted a rapid attainment of a stable $CD4^+$ T-cell set point which fails to explain the slow decline [317]. They proposed the model of virus adaptation, in which progression is driven by slow adaptation of the virus resulting in increased infectivity (e.g., co-receptor switch or changes in receptor expression on chronically stimulated target cells, viral fitness, immune escape), as a major force driving depletion [318].

5.3 HIV receptor complex signalling – impact on HIV replication

Although the introduction of highly active antiretroviral treatment has been a major breakthrough in the care of HIV-infected patients, it is not able to cure patients and is accompanied by poorly tolerable side effects and potential long-term toxicity. To develop new treatment strategies, more complete knowledge of the HIV replication cycle and its interactions with cell components and a detailed understanding of HIV immunopathogenesis are mandatory. In **chapter 4** we aimed to delineate motifs within the cellular receptor complex, CD4 and CCR5, that are critical for HIV replication and investigated the impact of cellular receptor complex-mediated signalling on several parameters of HIV replication (e.g., early reverse transcripts, productive infection, and virus production). In conclusion, our data suggest that CD4 signalling has an inhibitory effect on early HIV replication, whereas CCR5 signalling does not play a major role for this modulation, and that later steps of the HIV replication cycle are not directly affected by CD4/CCR5 signalling.

We also noted that, in some cases, mutation or deletion of signalling motifs in CD4 and/or CCR5 affected HIV replication in an isolate-dependent manner. This observation may have major negative implications for drug development: it will be highly unlikely that targeting a single host signalling molecule will have an impact on HIV infection since each HIV isolate will differently exploit this signalling pathway, some isolates will be inhibited while other isolates will show enhanced replication. This behaviour of HIV is reminiscent of the extreme viral versatility and ability of HIV to develop resistance to reverse transcriptase inhibitors.

We will pursue this line of research looking at the receptor function relationship of the receptor complex consisting of CD4 and CXCR4 and HIV replication. We would like to emphasize that CCR5-tropic strains are the strains sexually transmitted and which persist till late stage of HIV disease. The emergence of CXCR4-tropic strains is associated with a progressive decline of the HIV target cells, the $CD4^+$ T cells. We are convinced that the engagement of the HIV receptor complex by CXCR4-tropic strains is different from the one by CCR5-tropic strains and thus its molecular elucidation is critical for a detailed understanding of late stage HIV disease.

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7. Curriculum Vitae

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Publications

2006 **Baenziger S,** Tussiwand R, Schlaepfer E, Mazzucchelli L, Heikenwalder M, Kurrer MO, Behnke S. Frey J, Oxenius A, Joller H, Aguzzi A, Manz MG, Speck RF.

Disseminated and sustained HIV infection in CD34^{+} cord blood cell-transplanted Rag2$^{-/-}\gamma_c^{-/-}$ mice. PNAS 2006; 103(43): 15951-6.

Related Links:

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http://www.mediadesk.unizh.ch/mitteilung.php?text_id=146&grp=archiv

Article in the Unipublic Magazin (in german)
http://www.unipublic.unizh.ch/magazin/gesundheit/2006/2359.html

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CD4/CCR5 signalling affects HIV replication in an isolate dependent manner. (submitted)

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Triggering TLR7 in mice tesults in immune activation anddDisruption of the lymphoid system, resembling HIV-associated pathology. (submitted)

2008 **Baenziger S**, Ziegler P, Mazzucchelli L, Bronz L, Speck RF, Manz MG

Human T cell development and HIV infection in Human Hemato-Lymphoid System Mice. Current Topics in Microbiology and Immunology 2008, Vol.324. In press.

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03/2005 Speaker at the 4th day of clinical research, University Hospital Zurich.
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03/2005 Speaker at the 3rd Swiss Molecular Microbiology Workshop, ETHZ.
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05/2006 Speaker at the 2nd Swiss Workshop on Basic HIV research, Murten.

Sustained HIV replication and infection of lymphoid organs in cord-blood cell transplanted mice.

Poster at the 2nd Swiss Workshop on Basic HIV research, Murten.
CD4/CCR5 signalling affects HIV replication in an isolate-dependent manner.

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03/2007 Poster at KEYSTONE SYMPOSIA, Whistler, Canada.
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